

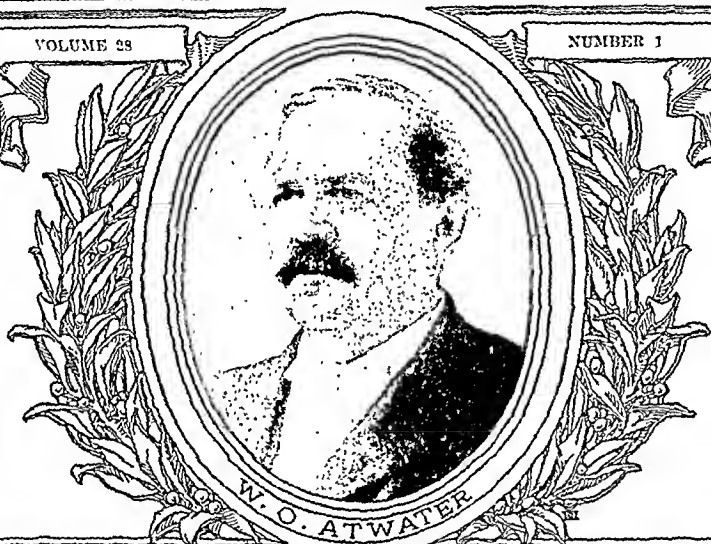


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# THE JOURNAL OF NUTRITION

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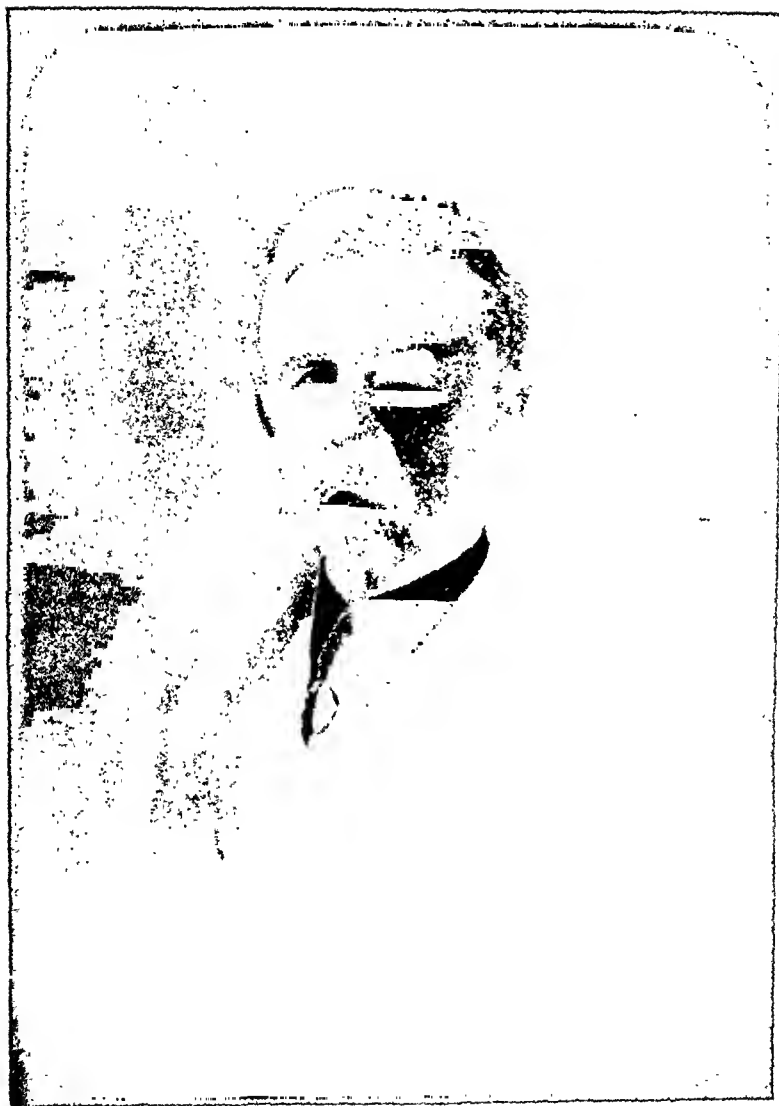
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*RUSSELL HENRY CHITTENDEN*

1856 - 1943

# RUSSELL HENRY CHITTENDEN

FEBRUARY 18, 1856-DECEMBER 26, 1943

## *An Appreciation*

Any account of the development of the science of nutrition must mention the name of Chittenden and the contributions he made during his 50 years of activity at Yale, the first 3 as a student, the next 7 as an instructor, and then 40 years as professor of physiological chemistry. He was the first man to hold such a post in America and to direct the first laboratory in this country devoted to this science. For 24 years he also served as Director of the Sheffield Scientific School, from which position he retired in 1922 when he had reached the age of 65. It was his good fortune to be with us for 23 years more and thus to be able to see in a long post-retirement period numerous striking developments in many phases of the science of physiological chemistry which he had helped to establish. He died on December 26, 1943. It is fitting that the pages of *The Journal of Nutrition* carry an appreciation of the contributions which Chittenden made as a scientist and of his traits as a teacher, and colleague.

Russell Henry Chittenden was born in New Haven, Connecticut, on February 18, 1856, of parents with a lineage going back to a William Chittenden who emigrated to this country from Cranbrook, Kent, England, in 1639. His early education was received in the New Haven public schools, and his preparation for Yale obtained in a local private school. An early interest in the classics eventually was replaced by one in natural science and the study of medicine which led finally to matriculation in the Sheffield Scientific School. He graduated at the age of 19 with a B.S. degree, having offered a thesis entitled "Glycogen and Glycocol in the Muscular Tissue of *Pecten irradians*." This was published in the *American Journal of Science and Arts* and subsequently translated into German and published in Liebig's *Annalen der Chemie*. The story of how this paper favorably influenced Kühne in 1878 to accept Chittenden as a student in the laboratory at the University of Heidelberg is well known to students of physiological chemistry who have read Chittenden's monograph "The Development of Physiological Chemistry in the United States" published 8 years after his retire-

ment from active duty at Yale.<sup>1</sup> The effect of his experience with Kühne was destined to be evident over a period of years in the numerous papers emanating from Chittenden's laboratory. It will be remembered that we owe to Kühne the word "enzyme" (in yeast), and that for many years Kühne was much interested in problems of digestion. Between 1875 and 1883, Chittenden's Laboratory of Physiological Chemistry published twelve papers; four more appeared in 1884, one of them written jointly with Kühne and published both in German in the *Zeitschrift für Biologie* and in English in the *American Chemical Journal*. Eleven more papers were published in 1885, seven of which dealt with various aspects of starch and protein digestion and may be regarded as reflecting the association with Kühne. One can go through all of the volumes of collected papers issued in those earlier years and find numerous examples of this continuing influence of Heidelberg.

Prof. Lafayette B. Mendel, one of Chittenden's pupils, frequently told his students how, in the few years prior to 1901 when Cohnheim announced the discovery of erepsin, Chittenden put many of his students to work on the problem of the fate in the body of the proteoses and peptones known to result from peptic and tryptic digestion. The failure to find these compounds in the blood led to the formulation of various hypotheses. One was that during absorption these compounds are combined in various ways to produce the proteins in the circulating blood. In vitro attempts were made to simulate the conditions prevailing in the intestines, such as peristalsis, movement of villi and the like. Mixtures of proteoses and peptones were placed in parchment bags, the bags immersed in appropriate fluids and then moved in various ways; after varying periods analyses were made of the fluids both inside and outside the bags. No evidence could be obtained supporting the idea of a recombination of proteoses and peptones to form larger protein complexes similar to those known to occur in the blood. The discovery of erepsin by Cohnheim in 1901 of course changed the entire point of view and approach to the basic problem, and focussed attention on the amino acids and relatively simple peptides.

Students of nutrition remember Chittenden chiefly for his work and ideas on the amount of protein needed for maintenance of the human adult. He was led to study this problem by the claims being made in 1902-03 by Mr. Horace Fletcher, who advocated that each mouthful of food should be chewed thirty-two times or more before being swallowed. Fletcher believed that by following his particular health regime he had found it possible to thrive on smaller amounts of food than most people commonly used. Chittenden took kindly to the idea that too

<sup>1</sup> R. H. Chittenden, "The Development of Physiological Chemistry in the United States," p. 33, American Chemical Society Monograph Series. Chemical Catalogue Co., New York, 1930.

great a consumption of protein with its attendant excretion of larger amounts of nitrogen metabolites through the kidney may mean imposing a greater physiological strain on the kidneys. As a scientist he saw that the problem might be studied through experiments aimed at determining the state of the nitrogen balance in men subsisting on low levels of protein intake. He therefore conducted nitrogen equilibrium experiments on himself and four of his colleagues, eight college students and thirteen volunteers from the U. S. Army. He found that nitrogen equilibrium could be maintained with a daily intake "one-half of the 118 gm. of proteide food called for daily by the ordinary dietary standards." The ordinary standard referred to here was the Voit standard. Chittenden also concluded that "body equilibrium can be maintained on far less than 3,000 cal. per day by the brain worker." These studies were reported in "Physiological Economy in Nutrition" published in 1905. They were then extended to include experiments on the dog, and reviewed in lectures given before the Lowell Institute in Boston in the early part of 1907 and published in the volume entitled "The Nutrition of Man."

Chittenden's distinguished position in the scientific world led to several interesting calls to public service. He was appointed by President Theodore Roosevelt to membership on the famous Remsen Referee Board asked to pass on the question whether sodium benzoate in foods is toxic. This board subjected Wiley's claim of toxicity to the rigors of laboratory experimentation and arrived at a negative answer. During World War I Chittenden served as a member of the executive committee of the National Research Council. During and after the war he and Graham Lusk visited Europe as representatives of the United States to meet with the Inter-Allied Scientific Food Commission. Lusk<sup>2</sup> has written how:

In the winter of 1918 Chittenden and I went to Europe . . . under instructions from our Government to reduce the food requisitions upon the United States to a minimum. The Food Committee of the Royal Society had adopted 3000 utilizable calories per day as the requirement of an average man doing an average day's work, and at the Paris meeting of the Inter-Allied Commission their representatives were inflexible in holding to this position. Before one of the meetings, while walking over the Pont Royal which took us to the left bank of the Seine, Chittenden said to me, 'Lusk, we are here to aid these suffering peoples to the maximum of our power.' A few minutes later he said before the startled commission, 'If you will not hear us we might as well go home.' This led to the unanimous adoption of a modification of statement that read: It was agreed that in case this ration could not be provided a reduction of not more than 10% could be borne for some time without injury to health.

<sup>2</sup> Graham Lusk, "American Contemporaries—Russell Henry Chittenden". Ind. Eng. Chem., vol. 21, p. 91 (Jan.) 1929.

Most of the present generation of students do not know of the part played by Chittenden in stimulating talented young men to enter medicine. Dr. John Howland, the noted pediatrician, stated that he received a greater stimulus from his course under Chittenden than was obtainable in the medical schools of that day. Harvey Cushing had planned to study architecture, but attendance on Chittenden's courses diverted him into medicine. In addition to Howland and Cushing Lusk cites the following list of great teachers as having received their greatest stimulus from Chittenden: Theodore Janeway, E. P. Joslin, Samuel W. Lambert, Richard P. Strong, Joseph A. Blake, John A. Hartwell, Lewis A. Conner, L. B. Mendel, A. N. Richards and Gideon Wells. Chittenden would persuade a student to spend an extra year with him after graduation and work in physiological chemistry. The effect of such a year, "shown by the record" as one might say, was certainly far reaching. As Lusk states it, "He built up the first true school of scientific endeavor concerned with the premedical education in this country; that is to say, he formed a group consisting of the master himself surrounded with pupils who in their turn became masters." His work was done in what was at one time the residence of Mr. Sheffield which had been converted into a laboratory. This old fashioned house, with one of its laboratories in what had been an art gallery and thus provided with a skylight, to mention but one of its interesting features, was Chittenden's workshop. During his years as Director of the Sheffield Scientific School, he relinquished more and more the work of active teaching of courses and direction of graduate students to his colleague, Prof. Lafayette B. Mendel. It was his good fortune to live to see his own work on the significance of protein in nutrition carried forward in the discovery of essential amino acids by Osborne and Mendel, and of the significance of these compounds in nutrition and physiological chemistry by a long list of workers too numerous to mention individually. He maintained a keen interest in these and related developments and was mentally alert until the very last. In his passing during his eighty-eighth year physiological chemistry and nutrition lost one of the American pioneers.

(G.R.C.)

# THE PRODUCTION OF HYPERCALCEMIA WITH SMALL AMOUNTS OF VITAMIN D<sup>1</sup>

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(Received for publication February 24, 1944)

Over a decade ago in publications from this laboratory (Jones et al., '30; Jones and Rapoport, '31) it was shown that the degree of hypercalcemia produced in dogs by large doses of vitamin D was dependent, to a large extent, on the amount of calcium in the diet. The hyperphosphatemia produced by excessive amounts of vitamin D, which is not so marked and is more irregular than the increase in serum calcium, appeared to be less dependent on the amount of dietary phosphorus. It was concluded that the net absorption of calcium from the intestinal tract was increased by the vitamin and there was less effect on the absorption of phosphorus. Harris and Innes ('31) observed that the toxicity of irradiated ergosterol was less on a calcium-low diet than on a diet containing liberal amounts of this element. Later, from studies on the fecal excretion of calcium and phosphorus, and on the absorption of these elements from isolated loops of the intestines, Nicholaysen ('37a, b), in agreement with our earlier work, concluded that vitamin D increased the absorption of calcium from the intestinal tract but had little effect on the absorption of phosphorus.

More recently Schneider and Steenhoek ('39) presented evidence to indicate that vitamin D acts by directing the phosphorus to the blood and growing bone at the expense of phosphorus which would otherwise be used by soft tissues. They based their conclusions upon the observation that vitamin D administered to rats on diets very low in phosphorus raised the level of inorganic phosphorus in the serum and increased calcification but reduced the rate of growth of the animals. They stated that the effect on growth was not due to toxicity of the vitamin as only small doses of the latter were given. They did not compare the levels of serum calcium in their vitamin-fed and control animals. Hypercalcemia invariably results from large doses of vitamin

<sup>1</sup> The data included in this paper were presented in abstract form in *Federation Proceedings* 1943, vol. 2, p. 64.

D and is thus a valuable guide in determining the extent of the toxic action of the vitamin. Small amounts of vitamin D have been found to produce a hyperealcemia when given to rats on the high calcium-low phosphorus type of rachitogenic diets (Jones, '38). Freeman and McLean ('41) have also reported that dogs on a diet containing very small amounts of phosphorus developed a marked hyperealcemia even when no vitamin D was given. In our work with diets very low in phosphorus we have repeatedly observed that vitamin D given at ordinary therapeutic levels frequently produces an appreciable hyperealcemia in rats. Following are the results of a detailed study of the factors concerned in the production of the hyperealcemia and the relation of the increase in serum calcium to changes in weight of the experimental animals.

#### EXPERIMENTAL

Albino rats were used as experimental animals and in the majority of cases were placed on the experimental diet at 25 days of age and continued on it for 21 days. At the end of this period the sera of several animals were pooled and analyzed for calcium by the method of Clark and Collip ('25) and for inorganic phosphorus by the method of Kuttner and Cohen as outlined by Youngburg and Youngburg ('30). One wrist bone from each animal was stained with silver nitrate and examined for the extent to which rickets had developed. The right femur was removed and the percentage ash determined on the lipid-free bone. The basal diet consisted of alcohol-extracted fibrin 18%, liver extract E<sup>2</sup> 2%, glucose (cerelose) containing 20 mg. of thiamine chloride per 100 gm. 1%, cottonseed oil 1%, and carotene-in-oil 3 drops per 100 gm. The salt mixture used in the basal diet was "salts 10" (Jones, '39) at a level of 3% which supplied about 0.33% calcium. When more calcium was desired it was furnished by additional calcium carbonate. Either dextrinized starch or glucose (cerelose) was added to make 100 gm. When any source of vitamin D was given it was dissolved in cottonseed oil at such a concentration that the desired amount was supplied by 1 gm. of the solution in 100 gm. of the diet. This solution replaced the cottonseed oil of the basal diet.

The above diet contains about 0.02% phosphorus. The diet used by Schneider and Steenbock ('39) contained 0.04% phosphorus. In some of the following experiments the phosphorus content of the diet was increased to equal that in the diet of Schneider and Steenbock by

<sup>2</sup> The liver extract was furnished by Dr. C. E. Graham of Wilson Laboratories, Chicago, Illinois.

replacing 1.5% of carbohydrate with an equal quantity of dried brewer's yeast. The calcium content of the diet used by these authors was 0.57%.

*Experiment I. 0.55% calcium, 0.04% phosphorus*

In the first experiment reported here (experiment 1, groups I and II, table 1) the phosphorus and calcium contents of the diet used were approximately equal to those of Schneider and Steenbock. In addition to the basal diet the animals of group I were given 100 I.U. of vitamin D per 100 gm. of diet. The data presented under the heading of group I are the averages obtained from five different trials done at different times. In three of the trials the vitamin D was furnished in the form of irradiated ergosterol<sup>3</sup> and in the other two experiments as pure crystalline calciferol.<sup>4</sup> Group II, which received no vitamin D, was composed of animals from the same litters as those used in group I. When comparing groups I and II it is seen that on the average the animals receiving the vitamin supplement gained about  $\frac{2}{3}$  as much in weight as the controls. The vitamin D, however, produced an increase in serum calcium of more than 2 mg. per 100 ml. of serum over that of the animals on the basal diet. The level of phosphorus, although still considerably below normal, was increased slightly by the vitamin. There was also an increase in calcification as shown by the weight and percentage of femur ash.

*Experiment II. 0.55% calcium, 0.02% phosphorus*

In the next experiment (experiment 2, groups III and IV, table 1) the conditions were kept the same except that the phosphorus was reduced to 0.02% by omitting the yeast. The results are very similar with the exception that the serum calcium of the vitamin-fed group (group III) was somewhat higher than in the corresponding group of the first experiment (group I). It is also to be noted that the animals of group III showed practically no growth during the experiment. The level of calcium in the serum of the animals on the diet without vitamin D was exceptionally high in this experiment. A certain degree of hyperealcemia invariably results in rats on the high calcium-low phosphorus diets, but the increase is usually not over 2 to 3 mg. per 100 ml. of serum.

<sup>3</sup> Kindly supplied by Mead Johnson and Company, Evansville, Indiana.

<sup>4</sup> Supplied through the courtesy of Dr. O. W. Barlow of Winthrop Chemical Company, Inc., Rennselaer, N. Y.



TABLE I  
Relation of dietary calcium to effect of vitamin D on growth, hypercalcemia,  
and calcification in rats on diets low in phosphorus.

EXP. NO.	GROUP NO.	NO. OF ANIMALS	DIETARY		VITAMIN		AVE. WT. ORNARD	SERUM			BONE ASH	
			Ca	P	Type	Amt. g		No. of analyses	Ca	P	mg.	%
1	I	18	0.55	0.04	I.E.†	100	14.4	5	14.5	3.3	23.7	28.3
1	II	17	0.55	0.04	.....	0	22.0	5	12.4	2.5	15.4	21.7
2	III	7	0.55	0.02	I.E.†	100	2.0	2	15.3	3.7	20.5	28.2
2	IV	5	0.55	0.02	.....	0	11.0	2	14.4	2.2	15.7	21.9
3	V	19	1.00	0.02	I.E.†	100	0.6⊕	5	15.9	3.1	26.4	28.9
3	VI	19	1.00	0.02	.....	0	9.1	5	12.3	1.3	16.7	21.5
4	VII	8	0.33	0.02	I.E.†	100	19.0	2	13.9	3.2	16.7	25.5
4	VIII	7	0.33	0.02	.....	0	13.4	2	10.8	1.9	14.0	20.5
5	IX	13	0.03	0.02	I.E.†	100	21.8	4	11.0	3.2*	15.1	22.3
5	X	13	0.03	0.02	.....	0	15.3	4	10.3	2.3	15.8	22.0
6	XI	10	1.00	0.01	I.E.†	100	4.5⊕	3	17.6	2.4	25.2	28.1
6	XII	9	1.00	0.01	.....	0	6.9	3	12.1	0.9	18.5	23.1
7	XIII	3	0.33	0.02	I.E.†	100	16.7	1	14.2	3.4	17.7	21.9
7	XIV	3	0.55	0.02	I.E.†	100	12.3	1	14.7	4.6	17.9	22.5
7	XV	3	1.00	0.02	I.E.†	100	7.0⊕	1	16.7	4.4	29.0	29.0
8	XVI	3	1.00	0.04	.....	0	11.0	1	14.5	1.5	20.0	25.7
8	XVII	4	1.00	0.04	D <sub>a</sub>	100	3.7	1	16.2	2.9	27.1	31.9
8	XVIII	3	1.00	0.04	A.T.-10	25*	20.7	1	14.0	3.0	20.4	27.3
8	XIX	3	0.33	0.04	A.T.-10	25*	23.3	1	12.7	2.7	21.8	26.1

† Irradiated ergosterol.

⊕ Three lots of animals given I.E. and 2 lots given crystalline calciferol.

⊖ International units of vitamin D per 100 grams of diet.

\* Micrograms per 100 grams of diet.

⊕ Loss.

*Experiment III. 1.00% calcium, 0.02% phosphorus*

To test further the relation of dietary calcium to the hypercalcemia and the inhibition of growth produced by vitamin D on a diet low in phosphorus, another experiment was conducted in which the phosphorus in the diet was maintained at a level of 0.02%, but the calcium was increased to 1.00%. The results (table 1, experiment 3) are but little different from those obtained in experiment 2. The animals in the group receiving the vitamin (group V), showed a very slight loss of weight during the experimental period, and the serum calcium was slightly above that of the corresponding animals of the previous experiment. In spite of the very low phosphorus content of the diet and a calcium to phosphorus ratio of about 50:1 there was an unmistakable increase in calcification of the femurs as shown by both the weight and percentage ash. In experiments 2 and 3 the average gain in weight of the animals not receiving the vitamin supplement was about 10 gm. for the entire period of 3 weeks. This compares with about 20 gm. for a similar length of time on rachitogenic diets somewhat higher in phosphorus.

*Experiment IV. 0.33% calcium, 0.02% phosphorus*

As there was only a slight difference in the results when the calcium was increased from 0.55% to 1.00% another experiment was carried out in which the calcium in the diet was reduced to 0.33%. The results are given in table 1 (experiment 4, groups VII and VIII). In this experiment the addition of vitamin D did not inhibit growth. In fact, the average gain in weight for the animals receiving the vitamin was somewhat more than that for the unsupplemented group. Similarly, Day and McCollum ('39), using a diet containing only slightly more calcium (0.40%) and low in phosphorus, did not find that the growth of their animals was inhibited by vitamin D. It is also to be noted that the serum calcium for group VII was less than for any of the above mentioned groups which received vitamin D, and calcification, especially as judged by weight of femur ash, appears to have been slightly less.

*Experiment V. 0.03% calcium, 0.02% phosphorus*

In the next experiment (experiment 5) a salt mixture containing no calcium was used in the basal diet and no calcium carbonate was added. This produced a marked inadequacy of calcium in addition to the deficiency of phosphorus. In spite of the low calcium to phosphorus ratio (approximately 1.5:1) these animals developed pro-

nounced rickets with a low serum phosphorus. The calcium deficiency was so severe that locomotion became difficult and many of the animals lost weight and some died before the end of the 21-day experimental period. In the experiment reported (table 1, groups IX and X) the length of time that the animals were maintained on the deficient diets was reduced to 14 or 16 days. On this diet the addition of the vitamin produced no hypercalcemia, and the animals showed a greater gain in weight than in any of the other experiments. In experiment 4 it was noted that calcification seemed to be somewhat less than in the experiments in which more calcium was given. In experiment 5 the addition of the vitamin apparently did not increase calcification to any extent as judged by the weight or percentage of bone ash. This was also indicated, but to a less degree, by examination of the wrist bones after staining with silver nitrate. The failure of calcification at the metaphyses in these animals cannot, however, be due solely to the low dietary calcium. It has been previously demonstrated (Jones and Cohn, '36) that the rachitic metaphyses of the rat can become calcified on a diet practically devoid of calcium if ample phosphorus is provided in which case the serum phosphorus of the rat is high. In the present experiment both calcium and phosphorus were extremely deficient resulting in a serum calcium below normal and a very low level of phosphorus. Under these conditions the vitamin was unable to increase the level of either element to the point where calcification would take place.

*Experiment VI. 1.00% calcium, 0.01% phosphorus*

In still another experiment (experiment 6, groups XI and XII) the phosphorus of the basal diet was reduced to about 0.01% by replacing the liver extract with synthetic vitamins of the B group.<sup>5</sup> The dietary calcium in this experiment was high. Again there was a marked increase in serum calcium in the vitamin-fed group, and the animals lost an average of 4.5 gm. in weight during the experimental period.

*Experiment VII. Effect of varying dietary calcium on response of litter mates*

Although the individual variations between different experiments in some cases are small, it is interesting to observe that in the series of experiments thus far reported (experiments 1 to 6) the levels of serum calcium in the vitamin-fed groups, without exception, are in

<sup>5</sup>100 gm. of the diet contained the following pure vitamins in the amounts indicated: Thiamine chloride 200  $\mu$ g., riboflavin 200  $\mu$ g., pyridoxine 200  $\mu$ g., pantothenic acid 1.2 mg., and nicotinic acid 1.2 mg. These compounds were all supplied by Merck and Company, Rahway, N. J.

reverse order to the gain in weight (average of each group) of the same animals. The relation of the amount of calcium in the diet to the degree of hypercalcemia produced by vitamin D and the relation of the hypercalcemia to growth is shown very well in experiment 7. In this experiment rats from the same litters were given varying amounts of calcium in addition to the basal diet containing 0.02% phosphorus. All animals were given the same amount of vitamin D. Here again the levels of serum calcium are in the same order as the amount of calcium in the diet and the changes in weight are in reverse order (table 1, groups XIII, XIV, and XV).

In the first experiment irradiated ergosterol and crystalline calciferol were used with no appreciable difference in results. In several trials the activated form of 7-dehydrocholesterol was used as the source of vitamin D in addition to a diet containing either 0.55% or 1.00% calcium and 0.04% phosphorus. In general the results were the same as when vitamin D<sub>2</sub> was used. That is, the vitamin produced a pronounced hypercalcemia and the extent to which growth was inhibited appeared to be related to the degree of hypercalcemia.

#### *Experiment VIII. Comparison of vitamins D<sub>3</sub> and A.T.-10*

Considerable evidence has been accumulated to indicate that per mg. dihydrotachysterol (A.T.-10) is as effective in producing a hypercalcemia under ordinary conditions as either vitamin D<sub>2</sub> or D<sub>3</sub> but is a poor antirachitic agent. Several experiments were carried out in which the effect of this material when given in connection with a diet very low in phosphorus was studied. In experiment 8 (table 1, groups XVI, XVII, XVIII and IX) the activated form of 7-dehydrocholesterol (vitamin D<sub>3</sub>) was compared with dihydrotachysterol in their effects on growth and the levels of serum calcium. The 7-dehydrocholesterol was a commercial preparation<sup>6</sup> which had been standardized in vitamin D units and, as is the case with irradiated ergosterol and calciferol, the dosage is expressed in units per 100 gm. of diet. The dihydrotachysterol was also a commercial preparation in which the activity was expressed in mg. of crystalline dihydrotachysterol per ml. of solution.<sup>7</sup> The dosage in this case is expressed in micrograms per 100

<sup>6</sup>The author is grateful to Dr. J. Waddell of E. I. duPont de Nemours and Company, New Brunswick, N. J., for the activated 7-dehydrocholesterol used in these studies.

<sup>7</sup>"Hytakerol" of Winthrop Chemical Company. According to the manufacturers this preparation contains the equivalent of at least 1.25 mg. crystalline dihydrotachysterol per ml. It contains 110-120 vitamin D units per ml., and 0.5 ml. raised the calcium in the serum of rats (200 gm.) to 14 mg. per cent.

gm. of diet. Assuming 40,000 I.U. per mg. to be the value of either  $D_2$  or  $D_3$ , the dose of 100 I.U. which was used in most of these experiments would be equivalent to 2.5  $\mu$ g. of the pure compound. Twenty-five  $\mu$ g. of dihydrotachysterol per 100 gm. of diet were given in experiment 9. In spite of this comparatively large dose there was little or no effect on either growth or the level of serum calcium. As usually measured, the hypercalcemic action of dihydrotachysterol, on a weight basis, is approximately equal to that of vitamin  $D_2$  or  $D_3$ , whereas its antirachitic action is much less. In the present case its hypercalcemic action is also much less. This would indicate that the ability of vitamins  $D_2$  and  $D_3$  to raise the serum calcium to abnormal values under the conditions described is associated with their antirachitic action in contradistinction to their more or less separate action as hypercalcemic agents.

#### DISCUSSION

The above data indicate clearly that the administration of vitamin D to rats on a diet high in calcium and very low in phosphorus produces a pronounced hypercalcemia, and the degree of the resulting hypercalcemia is dependent upon the amount of calcium in the diet. Furthermore, the inhibition of growth which accompanies the vitamin under these conditions is in turn dependent upon the hypercalcemia. It cannot be stated that the high level of serum calcium is the direct cause of the failure of growth. Schneider and Steenbock ('39) found no increase in concentration of inorganic phosphorus in various soft tissues of their animals during the experimental period whereas there was an increase in the ash content of the bones. They interpreted this observation as additional evidence in favor of their theory that vitamin D directs the phosphorus to the bones at the expense of the soft tissues. We were able to confirm these results on liver and muscle tissue taken from a few of the animals used in the experiments described above. However, in neither Schneider and Steenbock's nor our experiments was a decrease in the phosphorus content of the soft tissues observed as a result of the administration of the vitamin. Since normally there is no increase in the concentration of phosphorus (mg. of P per gm. of tissue) in the soft tissues during the growing period, the above results are what might be expected. Also, as the animals were not increasing in size, no absolute increase in the amount of phosphorus in these tissues would be anticipated. On the other hand calcification of bone could very well take place in a young animal that was not actually gaining in weight. This calcification unquestionably is aided by the vitamin. The data presented above give additional

evidence in favor of the view that vitamin D increases the absorption of calcium from the intestinal tract, and that the resulting calcification is dependent upon the level of both calcium and inorganic phosphorus in the serum. In the experiments in which a sufficient quantity of calcium was supplied in the diet the vitamin increased calcification although the level of serum phosphorus was still below what is generally regarded as the rachitic level. In contrast to this, when a diet lower in calcium was given, there was less calcification even though there was some increase in serum phosphorus. These observations serve to emphasize the importance of the interdependence of the levels in the serum of calcium and phosphorus in the process of calcification. Howland and Kramer ('22) were the first to recognize this relationship and evaluated it as the product of the concentrations of calcium and inorganic phosphorus expressed as mg. per 100 ml. of serum. Patwardhan and Sukhtankar ('43) have recently directed attention to this phase of the subject. Increasing the calcium-phosphorus product, by an increase in either the level of serum phosphorus or calcium would result in greater calcification. To this extent vitamin D might be considered as directing the phosphorus to the bone, but there is no evidence that the element is actually taken away from the soft tissues. In a previous publication from this laboratory (Jones and Rapoport, '31) it was shown that the nausea and vomiting in dogs caused by large doses of irradiated ergosterol were not related to the amount of vitamin D administered but to the resulting hypercalcemia. In the experiments reported above the failure of growth in rats again appears to be associated directly with the degree of hypercalcemia produced and is probably the result of the toxic action of the high serum calcium.

#### SUMMARY

The inclusion of one I.U. of vitamin D per gram in a diet very low in phosphorus and high in calcium produced a pronounced hypercalcemia in rats. Irradiated ergosterol, pure calciferol, or irradiated 7-dehydrocholesterol were equally effective, whereas dihydrotachysterol (A.T.-10) was much less effective. The degree of hypercalcemia produced by vitamin D was found to be dependent on the amount of calcium in the diet. Growth was inhibited only in those animals in which there was a rather marked hypercalcemia. In general, the extent to which growth was inhibited was directly related to the degree of hypercalcemia. Calcification, as judged by either the absolute or relative amounts of femur ash, was greater in those cases in which there was a definitive increase in serum calcium.

These data (1) give additional evidence in favor of the view that vitamin D increases the absorption of calcium from the intestines, and (2) serve to emphasize the importance of the calcium-phosphorus product of the serum in calcification.

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# THE EFFICIENCY OF UTILIZATION OF PHOSPHORUS BY THE ALBINO RAT<sup>1</sup>

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The purpose of this study was to compare the rates of absorption and utilization of phosphorus as present in phytin, in lecithin and in disodium phosphate; especially in relation to the pH of the alimentary contents and the phytin-splitting enzyme of the intestinal wall, and as the facts in these matters contribute to the understanding of the rachitogenic property of cereals.

Kinsman et al. ('39) gave a method for expressing the utilization of calcium in the following terms:

$$\frac{\text{Retention, Period A} - \text{Retention, Period B}}{\text{Intake, Period A} - \text{Intake, Period B}} \times 100 = \text{per cent utilization}$$

This method of representing efficiency of utilization was followed in the present investigation, the phosphorus intake in period A being high but not in excess of the level of maximum efficiency of utilization; and in period B the intake of phosphorus was just above the level of equilibrium.

The rachitogenic property of cereals was attributed by Bruce and Callow ('34) to the unavailability of the phytin phosphorus present. Harrison and Mellanby ('39) found that commercial phytin was not rachitogenic, whereas, sodium phytate was rachitogenic. They suggested that the rachitogenic action of cereals is normally due to the action of the phytic acid in inhibiting the absorption of calcium. Palmer and Mottram ('39) indicated that the unavailability of phytin phosphorus is associated with the disproportion of the levels of calcium and phosphorus fed.

Lowe and Steenbock ('36b) found that phytin phosphorus was absorbed although not as efficiently as inorganic phosphorus; but when the ration contained 3% calcium carbonate scarcely any of the phytin phosphorus was absorbed. They postulated that calcium carbonate

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inhibits the action of an intestinal enzyme which normally hydrolyzes phytin and makes its phosphorus available. In further studies Krieger et al. ('40a) comparing inositol-hexaphosphoric acid with inorganic phosphorus at a Ca/P ratio of 1:1, found the phytin phosphorus almost as readily utilized as the inorganic phosphorus. However, when the Ca/P ratio was 2:1 the availability of the phytin phosphorus was markedly decreased, while that of the inorganic phosphorus was not markedly affected. Jones ('39) also found that for rats the Ca/P ratio had a marked effect on the availability of phytin phosphorus. Krieger et al. ('40b) found the calcium of calcium phytate to be as available as that of calcium carbonate. McCance and Widdowson ('35) have shown that of the total phosphorus of cereals between 41 and 68% is phytin phosphorus, and that from 20 to 60% of the phytin fed to humans is excreted unchanged in the feces.

Eastman and Miller ('35), reporting on intestinal pH values, found the pH higher in rats fed a rachitogenic diet than in rats fed a normal diet.

Patwardhan ('37) found, in extracts from the intestinal wall of rats, an enzyme capable of the hydrolysis of sodium phytate. Patwardhan ruled out the possibility of the enzyme coming from the food because the enzyme was found present in weanling rats. Lowe and Steenbock ('36b) were of the opinion that any hydrolysis of the phytin which takes place in the intestines is due to the activity of the intestinal flora or to the phytase from the vegetable part of the diet.

#### EXPERIMENTAL

The experiment was carried out using growing albino rats as subjects. These animals were fed a commercial dog food<sup>2</sup> ad libitum until they reached a weight of about 80 to 90 gm. From this time on they received a basal ration in which phosphorus was present to the amount of 0.12%. This approximates the minimum amount for some growth. Following this basal period the rats were fed rations with double this amount of phosphorus, 0.25%. The extra phosphorus was in the form of disodium phosphate, of crude soy bean lecithin,<sup>3</sup> or of phytin.<sup>4</sup> In all cases the Ca/P ratio was approximately 2:1. The 0.12% phosphorus chosen for the basal ration was below the amount indicated by Forbes ('37) as being near the minimum, and not lower than the 0.113% indicated by Brown et al. ('32) as producing rickets.

<sup>2</sup> Purina dog chow.

<sup>3</sup> A. E. Staley Mfg. Co.

<sup>4</sup> Pfanstiehl Chemical Co.

For the higher phosphorus rations, 0.25% was chosen as being below the 0.257% indicated by Nicolaysen ('37) as that required for optimum growth.

The basal rations, per 1,000 gm., contained 60.0 gm. of wheat gluten, 120.0 gm. of lactalbumin, 538.1 gm. of corn sugar,<sup>5</sup> 80.0 gm. of a hydrogenated fat,<sup>6</sup> 70 gm. of butter, 26.0 gm. of calcium and phosphorus-free salt mixture (Sherman and Smith, '31), 30.0 gm. of cod liver oil, 64.0 gm. of milk vitamin concentrate,<sup>7</sup> 5 mg. of thiamine hydrochloride, 2.45 gm. of disodium phosphate, 2.68 gm. of calcium carbonate, and 6.77 gm. of sodium bicarbonate. The higher phosphorus rations had essentially the same composition except that the sodium bicarbonate was omitted and additional phosphorus was added as needed in the form of disodium phosphate, crude soy bean lecithin, or phytin. Since the crude lecithin contained some oil the amount of fat was cut down to 41.8 gm. for this ration. Calcium carbonate was added as needed to bring the C/P ratio to 2:1. In all cases the adjustment to 1,000 gm. of ration was made at the expense of the corn sugar. All rats were fed 7.14 gm. of ration per day.

Rats 1 to 36 used in basal period I and in period II were litter-mate triplets (table 1). In period II, on the higher phosphorus, one rat of the triplet received the disodium phosphate ration, a second the lecithin ration, and the third the phytin ration. Periods I and II were 14 days long with collection of urine and feces during the last 7 days in each case. Rats 37 to 64 used in basal period III and in period IV, were litter-mate pairs. In period IV, on the higher phosphorus, the first rat of the pair received the disodium phosphate ration and the second the phytin ration. Periods III and IV were 21 days long with collection of urine and feces during the last 14 days in each case.

In preparation for analysis the samples of feces, urine, or feed were digested with concentrated sulfuric-nitric acid mixture, and then made up to volume. Aliquots of these solutions were used to determine calcium by the McCrudden ('10; '11) method, and phosphorus by the Fiske and Subbarow ('25) method, using a photoelectric technique for the latter.

Following period IV the rats were gassed and the stomach, small intestine, and cecum were removed. The pH values of the contents of various segments were determined using a Beckman pH meter fitted with a glass electrode. Following this the small intestines and

<sup>5</sup> Cerelese.

<sup>6</sup> Crisco.

<sup>7</sup> Labco, no. 15-42.

cecum were freed of contents, washed, and ground in water containing chloroform. After autolysis, filtering, and dialysis, extracts were obtained, almost free of inorganic phosphorus, which contained an enzyme capable of splitting sodium phytate with the production of orthophosphate. This hydrolysis was accomplished by using 1 ml. of substrate (approximately 1% sodium phytate), 2 ml. of buffer (sodium diethylbarbiturate plus hydrochloric acid) at pH 7.7, and 1 ml. of enzyme extract. These solutions were analyzed for inorganic phosphorus at zero hours and after 20 hours at 38°C.

### RESULTS

In all four periods the rats showed no important differences between groups in the rate of body gain; but, as was expected, those in periods III and IV gained more because of the increased length of periods. The average weights of the rats, at the beginning of the basal collections, were from 97 to 101 gm.; and at the end of the higher phosphorus collection periods from 129 to 135 gm. for the shorter period, and from 148 to 152 gm. for the longer period.

Average balances of calcium and phosphorus are given in table 1. The percentages of calcium and phosphorus representing direct retention, and the percentages of calcium and phosphorus in the urine and feces, are given in table 2. Also given in table 2 are the percentages of "utilization" of calcium and phosphorus, using the Kinsman et al. ('39) formula. Table 2 also shows the weights of phosphorus gained in bone and soft tissue (98% of the calcium retained is in the bone, and this value is 2.15 times the weight of phosphorus in the bone). The weight of phosphorus retained in the bone was less for the phytin rats.

The average pH values of the contents of different parts of the alimentary tracts of the disodium phosphate rats were: stomach, 5.85; upper small intestine, 5.97; middle small intestine, 6.26; lower small intestine, 7.28; and cecum, 6.39. The corresponding values for the phytin rats were 5.53, 6.07, 6.38, 7.35, and 6.52. The average pH for the small intestine alone was 6.50 for the disodium phosphate rats and 6.60 for the phytin rats.

In the enzyme experiment, the freed inorganic phosphorus divided by the available phytate phosphorus was taken as the fraction hydrolyzed. The hydrolysis varied widely between 3.8% and 21.7%. Phytase was present in the extracts of both the disodium phosphate rats and the phytin rats, but no differences were noted between the groups.

TABLE I  
Average balances of calcium and phosphorus.

NUMBER OF RATS	RATION	INTAKE		OUTPUT						RETENTION		
		Ca mg.	P mg.	Urine		Feces		Total		Ca mg.	P mg.	
				Ca mg.	P mg.	Ca mg.	P mg.	Ca mg.	P mg.			
Period I Nos. 1-12 Nos. 13-24 Nos. 25-36	Basal	134.0	61.0	28.0	1.5	32.2	10.5	60.2	18.0	73.8	43.0	1.7
	Basal	134.0	61.0	25.6	1.6	35.6	19.7	61.2	21.3	72.8	39.7	1.8
	Basal	134.0	61.0	25.5	1.7	37.1	19.5	62.7	21.1	71.3	39.0	1.8
Period II Nos. 1-12 Nos. 13-24 Nos. 25-36	Basal + Na <sub>2</sub> HPO <sub>4</sub>	219.6	125.0	6.8	0.7	302.7	31.5	100.4	41.0	110.2	84.0	1.3
	Basal + lecithin	219.8	123.0	29.3	1.1	91.0	39.3	120.0	40.4	99.8	82.6	1.2
	Basal + phytin	218.8	115.5	44.6	1.2	94.3	47.0	138.9	48.8	80.7	66.7	1.2
Period III Nos. 37-50 Nos. 50-64	Basal	255.0	120.0	50.4	2.9	48.8	20.5	99.2	23.4	155.8	96.6	1.0
	Basal	253.7	119.4	53.0	2.9	47.1	20.2	100.0	23.0	153.7	96.4	1.6
Period IV Nos. 37-50 Nos. 50-64	Basal + Na <sub>2</sub> HPO <sub>4</sub>	461.0	241.0	3.8	39.0	163.3	52.1	167.2	89.9	293.8	151.1	2.0
	Basal + phytin	508.0	238.6	38.5	4.9	185.1	86.8	223.0	91.6	284.4	147.1	2.0

The higher average pH value of the intestinal contents of the phytin rats was associated with the higher average amount of phosphorus in the feces of these rats. The fact that the calcium content of the feces of both groups of rats was about the same, whereas, the fecal phosphorus of the phytin group was the higher, suggests that the higher intestinal pH was not optimal for the activity of the enzyme phytase. This would tend to limit the amount of phytin phosphorus available for absorption. This effect would be in addition to and not in place of the well-known effect of pH on the solubility of calcium-phosphorus salts. The higher intestinal pH of the phytin rats is in agreement with the higher pH values for rats on a rachitogenic ration reported by Eastman and Miller ('35).

The detection of an enzyme, phytase, in the extracts of the intestinal wall in both groups of rats agrees with the findings of Patwardhan ('37). However, there was no correlation between enzyme activity and utilization of phytin phosphorus. Such a correlation would not necessarily exist since the utilization of phytin phosphorus would be affected by conditions other than the freeing of this phosphorus from its organic combination.

#### SUMMARY

The utilization of phytin, lecithin and disodium phosphate phosphorus by growing albino rats was studied by means of balance experiments, efficiency of utilization being expressed as the difference in retention divided by the difference in intake.

In one experiment disodium phosphate phosphorus and crude soy bean lecithin phosphorus were utilized to the extent of 63.9% and 69.2%, respectively. Phytin phosphorus was utilized to the extent of 49.2%. The corresponding calcium utilizations were 41.9, 32.0, and 16.1%, respectively.

In another experiment of longer duration the disodium phosphate phosphorus was utilized to the extent of 45.0%, and the phytin phosphorus to the extent of 42.5%. This low utilization of disodium phosphate phosphorus was apparently due to a low level of calcium in the ration which made calcium the limiting element instead of phosphorus. The corresponding calcium utilizations were 67.0 and 51.5%, respectively.

The higher amount of phosphorus in the feces of the phytin rats apparently signified lower absorption.

The pH values for intestinal contents of individual rats varied much, but the average pH value, 6.60, for the phytin rats was higher than

that, 6.50, for the disodium phosphate rats. This higher average pH value for the intestinal contents of the phytin rats was associated with a lower utilization of phytin phosphorus.

The intestinal wall extracts for both the disodium phosphate rats and the phytin rats showed the presence of a phytate-splitting enzyme. No correlation was found between the enzyme activity of the extracts and the utilization of phytin phosphorus. However, the pH of the intestine may influence the activity of the phytase.

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# METABOLIC CHANGES IN GROWING CHICKENS<sup>1</sup>

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## TWO FIGURES

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The constancy of heat production per unit surface area in mature animals is better known than is the variability of this ratio in growing animals. The sequence of metabolic changes that occur during growth has not been determined in many species (for references see Kibler and Brody, '42) and the causal mechanisms which bring about these changes have not been segregated.

One approach to an analysis of these problems is a systematic study (daily measurements are necessary during the early stages of growth) of the changing metabolic levels in the same group of animals over the growing period. This paper reports such a study for the Rhode Island Red chicken.

## METHODS

Volumetric measurements of oxygen consumption were made individually on four male and four female birds with Regnault-Reiset type apparatus as previously used for measuring oxygen consumption in rats (Kibler and Brody, '42). Windows in the chambers permitted the observation of activity, and as readings were taken every 10 minutes for periods of 1 or more hours, observed activity could be eliminated from the results. After 2 months of age, oxygen consumption was measured after 24-hour fasts as well as after ad libitum feeding. Experimental temperatures were varied from 33°C. at hatching to 27°C. at later ages. The females were measured from hatching, July 30, 1942, to August 26, 1943 (one was killed accidentally before the end of the experiment). The males became too big for our larger apparatus when 6 months old, and so their measurements were discontinued at that time.

The birds were kept in wire bottom cages in a basement laboratory which was maintained at 75° to 85°F. Auxiliary overhead heaters

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permitted the chicks to partially control their own environmental temperature by moving closer to or farther away from the heating source. After hatching they were fed a standard chick-starter ration supplemented with buttermilk; this was followed by a growing mash and grain supplement as they became older.

#### ANALYSIS OF DATA

Mitchell, Card, and Haines ('27), using the Haldane gravimetric method and working principally with the White Leghorn breed, measured the basal metabolism of birds differing sufficiently in age to permit these authors to conclude that the metabolism per unit of body surface is distinctly below the adult level at hatching, rises rapidly to a maximum between 30 to 40 days of age and then decreases again to adult level at about 70 to 80 days of age.

Our measurements on the growing rat ('42) suggested the possibility of correlating changes in metabolism per unit surface with percentage changes in normal growth rate. For our purpose, therefore, it seemed more desirable to measure the metabolic changes associated with age in the same "normally" fed chickens rather than to measure the metabolism of fasted birds of different ages. Accordingly, all our measurements during the first 2 critical months after hatching were made on non-fasted chicks at rest at a temperature of thermoneutrality.

The non-fasting heat production per square meter per day during this critical period increased from about 750 Cal. at hatching to a maximum of 1,250 to 1,300 Cal. at 4 to 5 weeks of age, and then decreased to about 900 Cal. at 8 weeks (upper left-hand section of fig. 1). A value of 920 Cal. (same section of figure) was reported by Mitchell et al. ('27) as an average basal value for 55-day-old chicks of this breed.

The metabolism per unit surface in the older birds became relatively stable as shown by the 24-hour fasting and non-fasting curves (right hand section, fig. 1). Intermittent egg production apparently caused some irregularity in the data for the females. The high values for the large males are probably due, in part, to the fact that they became too large to be comfortable in the chambers of our apparatus. However, Mitchell and Haines ('27) also obtained higher basal values in the males than in the females of this breed. Their average basal values for mature groups of each sex are plotted in the lower right-hand section (fig. 1).

When plotted against age, sex differences in metabolism per unit of surface area are minor (upper left-hand section, fig. 1), but when plotted against body weight (lower left-hand section, fig. 1) sex differ-

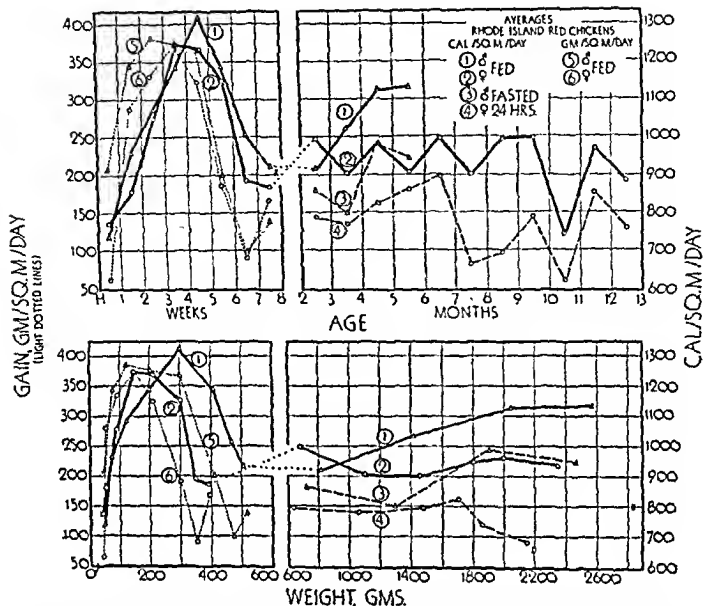


Fig. 1 Metabolism per unit surface area as a function of age (upper section) and of weight (lower section). In addition to our curves, scattered basal data from the literature (Mitchell et al., '27) were averaged into three points and plotted for comparison as follows: the open rectangle represents mature females, the solid rectangle mature males, and the X, 55-day-old chicks (sex not recorded). Light dotted curves representing growth rate per unit surface area are likewise plotted in the left-hand section and attention is called to the apparent parallelism between these curves and the corresponding curves for heat production per unit of surface area.

ences are quite evident, the females attaining maximum heat production per square meter of surface at 150 gm. and the males at 300 gm.

When total metabolism is plotted against body weight on logarithmic paper (fig. 2), the data appear to be distributed in three segments corresponding to the rising, the declining, and the mature stages of figure 1.

To each of these segments, the equation  $Y = aX^b$  was fitted to relate total metabolism to body weight. The slope of each fitted curve, or the value of exponent  $b$ , measures the relative or percentage rate of increase in metabolism  $Y$  as compared to that in body weight  $X$ . Sta-

tistical constants are tabulated in the upper left-hand corner of figure 2. Rho represents the correlation coefficient and  $S_R$  represents the standard error of estimate. Two-thirds of the plotted individual measurements for each equation are included between plus and minus  $S_R$ .

In the segment representing the age period, hatching to 5 weeks, slope  $b$  is approximately 1.00, meaning that the percentage rate of increase in metabolism is as rapid as that in body weight at any time during this period, or that metabolism is directly proportional to body weight during this period.

In the final stage (2 months of age and over) the metabolism increased at a somewhat lower rate relative to body weight; slope

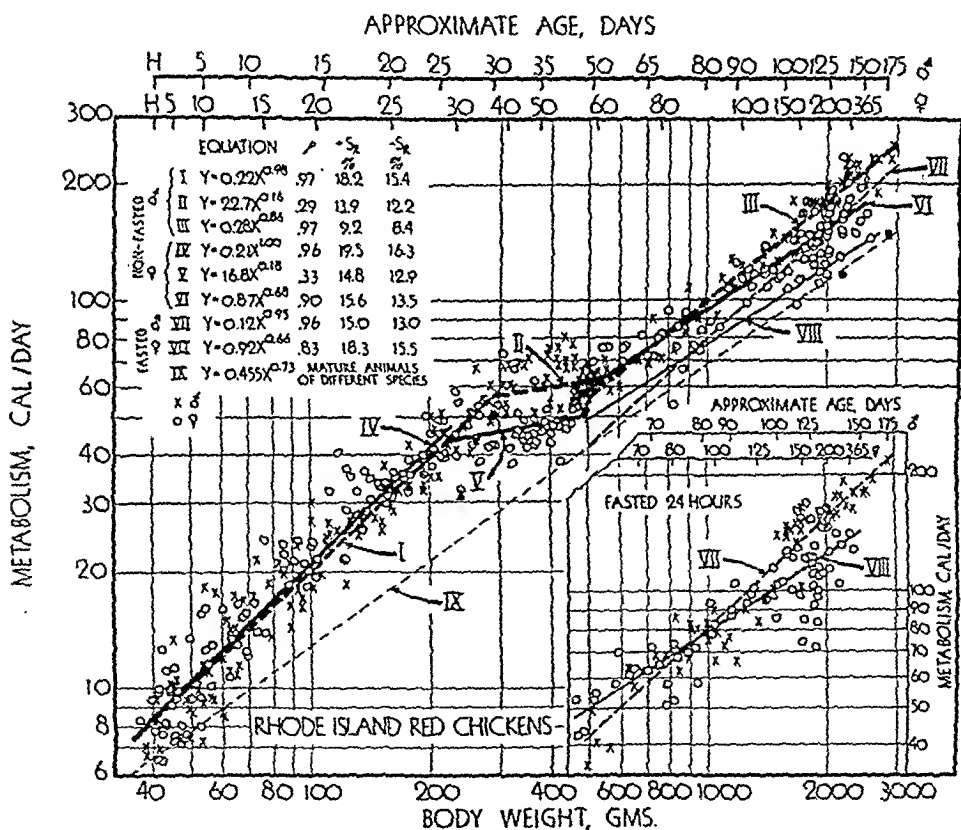


Fig. 2 Metabolism as a function of body weight (individual data plotted on logarithmic paper) with age estimated (upper axis). The 24-hour fasting data are shown in insert. Equations and statistical constants (obtained by the method of least squares) are tabulated for the various stages of growth. Equation IX for mature animals of different species is taken from Brody, Procter, and Ashworth ('34) and the solid triangle, circle, and rectangle represent, respectively, 55-day-old chicks (sex not recorded), mature females, and mature males from Mitchell et al. ('27).

$b = 0.97$  in the rapidly growing males and  $0.68$  in the less rapidly growing females. For the 24-hour fasting data for this stage,  $b = 0.96$  for the males and  $0.83$  for the females.

Between the initial and final stages there is a short interval, corresponding to the declining stage of figure 1, in which heat production increases very little with increasing body weight. The data during this period are somewhat erratic and the equations which we fitted as a matter of routine have low, though significant, coefficients of correlation (see values of  $\rho$  in upper left-hand corner of fig. 2). We do not, therefore, attach much significance to the numerical value  $0.2$  which we calculated for slope  $h$ .

As a matter of fact, we do not attach very great biological significance to the exact numerical values of slope  $h$  which we found for any of the stages, although the coefficients of correlation are quite high in most cases. Titus and Jull ('28) have pointed out the "great difficulty if not impossibility of setting up a normal growth curve" for all strains of a given breed of chickens and if, as we have suggested, metabolic rate is correlated with growth rate, it is, perhaps, likewise impossible to set up a "normal" curve of metabolism for all growing Rhode Island Red chickens.

The position of curve IX (fig. 2) for basal metabolism of mature animals of different species from Brody, Procter and Ashworth ('34) indicates that metabolism in chickens compared to that in mature animals of different species is high only during the period of rapid growth.

The possible correlation of changes in percentage growth rate with changes in metabolism per unit surface (fig. 1) or with changes in slope  $h$  (fig. 2) could be discussed in detail as was done in a previous report on the growing rat ('42), but a more understandable physical relationship between heat production and growth rate may be derived by relating both heat production and growth rate to surface area.

Assuming that rapid growth rate is associated with high heat production, then the more rapid the growth rate, the greater must be the rate of heat dissipation through each unit of surface. To test this hypothesis we have computed (table 1) heat production in Calories per day per square meter and growth rate in grams per day per square meter.

A comparison of the two columns (table 1) indicates that both heat production in Calories per square meter per day and growth rate in grams per square meter per day are higher from the third to fifth weeks than at any other age. It is true that the males gained an average of 25 gm. per day during the third month compared to a gain of 12.6 gm.

per day during the third week, but per square meter of surface these gains amounted to 377 gm. per day in the third week compared to 188 gm. per day in the third month.

To further test the validity of our hypothesis that heat production per unit surface and growth rate per unit surface are related, we

TABLE 1  
*Growth and metabolism of Rhode Island Red chickens.  
(24-hour fasting data given on starred lines.)*

AGE PERIOD	FEMALES					MALES				
	Average per chicken					Average per chicken				
	No. observations	Body weight	Cal. <sup>1</sup> per day	Cal./sq.m. <sup>2</sup> per day	Gm./sq.m. <sup>2</sup> per day	No. observations	Body weight	Cal. <sup>1</sup> per day	Cal./sq.m. <sup>2</sup> per day	Gm./sq.m. <sup>2</sup> per day
<i>Week</i>										
H-1	19	43	8.8	774	62	22	48	9.1	740	209
1-2	26	54	11.6	862	280	28	74	16.1	965	346
2-3	22	91	20.4	1056	335	26	124	26.0	1086	384
3-4	26	152	34.5	1250	375	23	201	40.2	1197	377
4-5	21	216	43.8	1240	324	21	294	58.2	1328	368
5-6	14	299	51.2	1155	189	16	410	65.9	1191	201
6-7	8	351	44.3	893	91	12	471	61.5	1009	99
7-8	17	392	47.0	877	168	21	509	60.3	937	141
<i>Month</i>										
2-3	16	679	78.8	1001	184	13	821	85	920	191
*2-3	16	631	59.4	794	...	16	728	63	866	...
3-4	14	1152	104	913	140	14	1436	138	1037	188
*3-4	12	1048	83	778	...	18	1300	100	806	...
4-5	6	1602	142	992	70	17	2023	190	1125	98
*4-5	9	1479	113	834	...	13	1914	161	990	...
5-6	9	1724	139	917	...	9	2580	229	1143	...
*5-6	3	1319	109	872	...	7	2470	185	952	...
6-7	6	1784	156	1008	...	..	....	....	....	...
*6-7	4	1679	134	906	...	..	....	....	....	...
7-8	9	1925	149	913	...	..	....	....	....	...
*7-8	9	1822	106	674	...	..	....	....	....	...
8-9	9	2126	176	1003	...	..	....	....	....	...
*8-9	4	1864	112	705	...	..	....	....	....	...
9-10	7	2054	172	1008	...	..	....	....	....	...
*9-10	8	1944	131	798	...	..	....	....	....	...
10-11	5	1878	121	755	...	..	....	....	....	...
*10-11	6	1818	99	633	...	..	....	....	....	...
11-12	6	1931	161	983	...	..	....	....	....	...
*11-12	6	1835	142	865	...	..	....	....	....	...
12-13	4	2260	164	897	...	..	....	....	....	...
*12-13	4	2084	113	773	...	..	....	....	....	...

<sup>1</sup> The heat production was calculated on the assumption that 1 liter of oxygen has a heat equivalent of 4.9 Cal. for the fed chickens and 4.7 Cal. for the fasted chickens.

<sup>2</sup> Surface area was computed from the equation, surface area in sq. cm. = 8.19 (body weight in grams)<sup>0.705</sup> as determined for White Leghorn chickens by Mitchell ('30).

plotted in figure 1, left-hand section, the changes in growth rate per square meter. The resulting inverted U-shaped curves are similar to the curves for heat production per square meter, except that the growth curves pass through corresponding phases at slightly earlier ages and lower weights than the heat production curves.

It might be inferred from our data, or from those of Mitchell et al. ('27), that heat production per unit of surface is always at a maximum at 30 to 35 days after hatching. Such may or may not be the case. If heat production is highest during most rapid growth, then maximum heat production per unit surface will occur early in accelerated early growth and late in accelerated late growth. Further metabolic measurements on two groups of chickens will be needed to determine this point.

Maximum growth in weight per unit of surface certainly does not always occur at the same age. Titus and Jull ('28) reported weekly body weight data on two lots of Rhode Island Red chickens which received the same treatment and feed, with the one exception that lot 2 had access to sour skim milk and water, while lot 1 received only water. They reported that feeding skim milk accelerated growth during the first 15 to 18 weeks, but had no marked effect on calculated mature weights. When we computed the gains per day per square meter made by their two lots of chickens (computed separately for males and females in each lot), we found that the milk-fed lot made maximum gains from the second to sixth week, whereas the other lot made their greatest gains from the fifth to the ninth weeks. Our chicks were given buttermilk and grew as rapidly as their rapid growth lot up to 5 weeks, but did not grow so rapidly thereafter, especially during the seventh week. Our birds were heavier at all ages, however, than the slow growth lot of Titus and Jull.

While, therefore, our analysis demonstrates an apparent parallelism between metabolic rate and growth rate, it does not segregate the nutritional, endocrine, and other causal mechanisms. For this purpose it would be helpful to determine the degree of parallelism between the growth rate and metabolic rate of several groups timed to reach their maximal growth rates at different ages.

#### SUMMARY

Data are reported on oxygen consumption in relation to body weight, age, and surface area of Rhode Island Red chickens from hatching to 6 months of age in males and to 13 months of age in females.

Attention is directed to an apparent parallelism during the first 2 months after hatching, between metabolism per day, expressed in

Calories per square meter, and growth per day, expressed in grams per square meter. The resting but non-fasting heat production at thermoneutrality increased during this critical period from about 750 Cal. at hatching to a maximum of 1,250 to 1,300 Cal. at about 30 days, and thereafter decreased to 900 Cal. at 60 days. Daily growth rate per square meter similarly increased to a maximum of approximately 370 gm. at about 25 days, and thereafter decreased.

After 2 months of age, the non-fasting heat production in the females averaged about 900 Cal. as compared to 750 Cal. after 24-hour fasts. Higher values were obtained for the males.

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# VITAMIN CONTENT OF VARIETY MEATS<sup>1</sup>

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In previous publications, McIntire et al. ('43a), Schweigert et al. ('43), Schweigert et al. ('43a) and McIntire et al. ('43), the vitamin content of pork, veal and lamb has been studied. Due to the increasing use of variety meats during the present emergency it is important to have a knowledge of their vitamin content. The results of the vitamin analyses of a selected group of these meats are reported in this paper.

## EXPERIMENTAL

The meats were obtained from local markets and prepared immediately for vitamin analyses and cooking tests. All samples were ground in an electric meat grinder and mixed thoroughly preparatory to vitamin assays. The samples that were used for cooking were prepared as follows: Frankfurters and pork links were obtained in pairs; one link from each pair was used for fresh analysis and one was retained for cooking tests. Canadian bacon and liver were prepared by slicing, and alternate pieces were taken for fresh and cooked samples. Bologna and veal hearts were cut into halves and alternate halves were taken for fresh and cooked samples; the halves to be cooked were sewed tightly together. Three baby beef hearts were taken from animals from a herd of the same age and breed. Two of the hearts were cut into halves and alternate halves were used for fresh and cooked samples; two halves were sewed together before cooking. The third heart was cooked whole to compare with the paired sample. The three tongues were prepared in the same manner.

All cooking tests were carried out under standard conditions according to Meat and Meat Cookery ('42). Frankfurters were boiled for 8 minutes; the bologna was boiled for 20 minutes and the two tongues were boiled for 3 hours 21 minutes and 2 hours 44 minutes respectively.

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Pork links were broiled for 17 minutes; Canadian bacon was broiled for 11 minutes; baby beef liver was broiled for 14 minutes. Beef liver and veal heart were braised for 38 minutes and 2 hours 50 minutes, respectively, and the two beef hearts were braised for 5 hours 12 minutes and 5 hours 30 minutes, respectively. The drippings of all cooked meats except for boiled frankfurters and bologna were retained for analysis.

The thiamine determinations were made according to the method of Hennessy ('42) with modifications by McIntire et al. ('43a). The nicotinic acid was determined by the method of Snell and Wright ('41) with modifications by Krehl et al. ('43) after extraction with 4% NaOH. Both fluorometric and microbiological methods were employed

TABLE 1  
*Comparison of methods for riboflavin determination.*

MEAT	FLUOROMETRIC ANALYSIS	MICROBIOLOGICAL
	mg./100 gm.	mg./100 gm.
Bologna (fresh)	.21	.21
Bologna (boiled)	.22	.20
Frankfurters (fresh)	.17	.17
Frankfurters (boiled)	.16	.16
Pork links (fresh)	.15	.15
Pork links (broiled)	.21	.24
Baby beef liver (fresh)	2.45	2.40
Baby beef liver (broiled)	3.43	3.03
Beef liver (fresh)	3.39	3.10
Beef liver (braised)	4.47	4.33
Veal heart (braised)	1.15	1.15
Canadian bacon (fresh)	.17	.19
Canadian bacon (braised)	.25	.24

for the determination of riboflavin. All of the riboflavin determinations were made by the fluorometric method after values were found to agree with those obtained by the microbiological method (table 1). The microbiological method was that of Snell and Strong ('39) with modifications in extraction and digestion (Strong and Carpenter, '42; McIntire et al., '43). The fluorometric method was that of Connor and Straub ('41), modified by Andrews ('43).

All analyses were made on the undried cooked and uncooked meat. The values for all of the samples assayed are shown in table 2. Retention of the vitamins in the meat was determined by the difference in the total vitamin content in a piece of meat before and after cooking.

Total retention represents the difference in the total vitamin content of the fresh meat and the cooked meat plus the drippings. The percentage of the vitamins retained after cooking is shown in table 3.

TABLE 2

## Vitamin content of meats.

All values in milligrams per 100 gm. of undried meat.

MEAT	THIAMINE	RIBO- FLAVIN	NICO- TINIC ACID	MEAT	THIAMINE	RIBO- FLAVIN	NICO- TINIC ACID
Bacon				Oxtail	.07	.16	2.8
.....	.19	.10	.8	Lamb shank	.14	.18	4.5
Canadian <sup>1</sup>	.57	.18	2.8	Liver			
Broiled <sup>1</sup>	.74	.24	4.1	Baby beef <sup>1</sup>	.19	2.43	16.9
Bologna				Broiled <sup>1</sup>	.19	3.29	19.2
.....	.22	.39	3.3	Beef <sup>1</sup>	.23	3.32	13.5
..... <sup>1</sup>	.30	.21	2.8	Braised <sup>1</sup>	.19	4.42	13.6
Boiled <sup>1</sup>	.20	.21	2.5	Heart			
Frankfurters				Lamb	.45	.74	6.3
.....	.31	.31	2.7	Veal	.70	1.00	7.0
..... <sup>1</sup>	.25	.17	2.6	..... <sup>1</sup>	.56	1.10	6.6
Boiled <sup>1</sup>	.24	.16	2.4	Braised <sup>1</sup>	.20	1.15	5.7
Sausage				Baby beef <sup>1</sup>	.40	.76	4.5
Summer	.46	.36	4.1	Braised <sup>1</sup>	.11	.92	3.7
Liver	.20	1.30	5.7	Braised <sup>1</sup>	.16	.91	3.6
Salami	.25	.21	2.9	Tongue			
Links				Baby beef <sup>1</sup>	.16	.28	3.0
Pork <sup>1</sup>	.40	.15	2.3	Boiled <sup>1</sup>	.05	.29	2.6
Broiled <sup>1</sup>	.50	.22	3.0	Boiled <sup>1</sup>	.05	.27	2.8
Tripe	..	.15	1.6	Beef			
Head cheese	.08	.12	1.1	Corned	.05	.19	1.7
Sandwich meat	.43	.18	3.6	Tenderloin	.15	.35	3.5

<sup>1</sup> Samples used for cooking tests.

TABLE 3

## Vitamin retention after cooking.

MEAT	MODE OF COOKING	WEIGHT RETENTION	THIAMINE RETENTION		RIBOFLAVIN RETENTION		NICOTINIC ACID RETENTION	
			Meat	Total	Meat	Total	Meat	Total
Bologna	Boiled	100	97	..	98	...	89	...
Frankfurters	Boiled	101	97	..	98	...	92	...
Pork links	Broiled	60	75	87	83	90	78	92
Canadian bacon	Broiled	63	94	98	88	92	04	93
Baby beef liver	Broiled	75	77	91	101	108	85	100
Beef liver	Braised	69	58	84	92	109	70	98
Veal heart	Braised	69	25	41	74	99	60	95
Baby beef heart	Braised	63	22	31	76	96	51	82
Tongue	Boiled	80	24	33	79	100	55	92

## DISCUSSION

The variety meats may be important sources of vitamins. Canadian bacon, summer sausage, and sandwich meat contain larger amounts of thiamine than fresh beef, veal or lamb but smaller amounts than fresh pork. Corned beef was the poorest source of thiamine; this is to be expected due to the source and processing. Of the fresh organ meats studied heart was found to be the richest in thiamine. Two veal hearts contained 0.7 and 0.56 mg. of thiamine per 100 gm. respectively; lamb heart 0.45 mg.; and baby beef heart 0.4 mg. per 100 gm. Liver and tongue contained about the same amount of thiamine as muscle meat. Beef liver contained 0.23 mg.; baby beef liver 0.19 mg., and baby beef tongue 0.16 mg. per 100 gm. These values are in good agreement with those reported by Cheldelin and Williams ('42), namely, 0.26 mg. of thiamine per 100 gm. of beef liver and 0.44 mg. per 100 gm. of beef heart.

Liver sausage contains nearly 3 or 4 times as much riboflavin as any of the other prepared meats. This is undoubtedly due to the high liver content. The other prepared meats fall in the same range as fresh muscle meats; corned beef contained the least riboflavin.

In the studies on the organ meats liver was found to be the best source of riboflavin. Beef liver contained 3.3 mg. and baby beef liver 2.4 mg. per 100 gm. of meat. Heart was a good source of riboflavin; veal heart contained 1.1 mg. per 100 gm. and baby beef heart contained 0.76 mg. per 100 gm. The riboflavin content of tongue was 0.28 mg. per 100 gm. These values are in agreement with those reported in the literature. Cheldelin and Williams ('42) reported 2.8 mg. per 100 gm. of fresh liver and 0.88 mg. per 100 gm. of beef heart. Waisman and Elvehjem ('41) reported 0.76-1.0 mg. of riboflavin per 100 gm. of beef heart and 2.1 and 3.7 mg. per 100 gm. of beef liver, and 0.22 mg. of riboflavin per 100 gm. of beef tongue.

The nicotinic acid contents of the prepared meats and fresh muscle meat are about the same. Liver was richer than any of the organ meats in nicotinic acid. Baby beef liver contained 16.9 mg. per 100 gm. of meat and beef liver contained 13.5 mg. Heart was lower, but it is also a good source of nicotinic acid. Lamb, veal and baby beef heart contained 6.3, 7.0, and 4.5 mg. of nicotinic acid per 100 gm. of meat. Our values for nicotinic acid generally agree with those reported by other workers. Dann and Handler ('42) reported 5.8 to 7.7 mg. of nicotinic acid per 100 gm. of lamb heart and 11.5 to 15.6 mg. of nicotinic acid per 100 gm. of veal liver. Cheldelin and Williams ('42) reported 12.0 mg. per 100 gm. of beef liver. Waisman and Elvehjem ('41) reported

13.2 mg. of nicotinic acid per 100 gm. of veal liver, 4.9 mg. per 100 gm. of beef heart and 8.5 and 11.0 mg. per 100 gm. of beef liver.

Considerable variation in the retention of the vitamins during cooking was obtained. This may be attributed to the type of cookery, the size and kind of meat and the length of time necessary for cooking. To illustrate the effect of time and kind of meat on vitamin retention, bologna, frankfurters, and tongue were boiled. The frankfurters and bologna were in the cooking water 8 and 20 minutes, respectively, and negligible vitamin loss occurred. However, tongues that were cooked between 2 and 4 hours retained only 24% of the thiamine in the meat and only 33% total retention was observed. The fact that only 55% of the nicotinic acid remained in the meat whereas 37% was recovered in the drippings illustrates the degree of leaching that occurs under such cooking conditions. Similar results during stewing of veal and lamb have been obtained by McIntire et al., '43.

We have previously reported that broiling meat results in higher retention of the thiamine than any other type of cookery studied. This was found to be the case in the present investigation. Pork links, Canadian bacon, and baby beef liver after broiling retained 75, 94 and 77% of the thiamine in the meat, respectively. On the other hand, when beef liver, veal heart and baby beef heart were braised, 56, 25 and 22% of the thiamine, respectively, were retained. The very low retention of thiamine in the hearts may be accounted for by the length of time required for cooking.

Similarly there was a higher retention of riboflavin and nicotinic acid in the meat after broiling than after braising, but, in every case except one, over 90% of these vitamins was recovered in the meat and drippings.

It should be pointed out that uniform sampling of single organs such as heart and tongue is extremely difficult. By cutting these organs in half and sewing the halves together for cooking tests a fair sample was obtained. For comparison a whole organ which was taken from an animal of the same breed, age and nutritional background gave similar results. This, however, should not be taken as an indication that organs from different animals have the same vitamin content in every case.

#### SUMMARY

The thiamine, riboflavin and nicotinic acid content of a variety of meats has been determined.

Prepared meats were found to be a good source of these vitamins; they contain about the same amounts as fresh muscle meats.

## DISCUSSION

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#### SUMMARY

The thiamine, riboflavin and nicotinic acid content of a variety of meats has been determined.

Prepared meats were found to be a good source of these vitamins; they contain about the same amounts as fresh muscle meats.

Retention of these vitamins in some of the meats was studied during broiling, braising and boiling.

Greater amounts of all the vitamins were retained in the meat after broiling than after braising.

In the case of boiling the vitamin retention in the meat was dependent on the cooking time.

Broiling favored a higher total retention of thiamine than did braising.

In nearly every case over 90% of the nicotinic acid and riboflavin was recovered in the meat and drippings.

#### ACKNOWLEDGMENTS

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# THE PROTEIN NUTRITIONAL VALUE OF SOYBEAN, PEANUT, AND COTTONSEED FLOURS AND THEIR VALUE AS SUPPLEMENTS TO WHEAT FLOUR<sup>1</sup>

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Increased interest has recently developed in the nutritive value of plant proteins that may serve in the diet to extend and partially replace proteins of animal origin, particularly those of meat, milk, and eggs. Soybeans, peanuts, and cottonseed offer excellent possibilities for supplying in large quantities proteins having superior nutritive value and which are suitable for use as human food in a variety of ways. Heat processed soybean flour and grits for human consumption are being produced in large quantities. Peanut and cottonseed flours in limited amounts are also available.

Numerous investigations have been conducted on the nutritive value of soybean proteins. Mendel and Fine ('12) found that soybean nitrogen was well utilized by men and dogs. Later, Osborne and Mendel ('17a) showed that the proteins of soybean meal supported "normal growth" of rats, provided that the meal had been adequately heated with water. Raw meal enabled the animals to grow at a comparatively slow rate. Heating the meal in an electric oven at 110°C. for 4 hours, however, caused no considerable improvement. More recently, others (Wilgus, Norris, and Heuser, '36; Hayward, Steenbock, and Bohstedt, '36a, b; Shrewsbury and Vestal, '37; Johnson, Parsons, and Steenbock, '39) have shown that the proteins of heat-treated soybeans have a high nutritive value. Soybean proteins have also been found to be an effective supplement to the proteins of wheat flour (Johns and Finks, '21; Johns, Finks, and Jones, '23; Daniels and Nichols, '17).

Studies on the nutritive value of the proteins of peanuts (Johns and Finks, '20; Eddy and Eekman, '23; Daniels and Loughlin, '18)

<sup>1</sup>A preliminary report of this investigation was given at the 22nd Annual Convention of the American Soybean Association, Purdue University, September 15-17, 1942.



have shown that the peanut also contains proteins of high quality and are valuable supplements to cereal proteins.

Cottonseed meal has been highly esteemed for many years as a protein concentrate for livestock feeding. The nutritive value of the proteins of cottonseed was studied by Osborne and Mendel ('17b). They state that their results leave "no doubt as to the adequacy of the proteins of the cottonseed meal or flour for the growth of rats." Recently, Zucker and Zucker ('43) have shown that cottonseed and also peanut and soybean are sources of high quality protein.

Most of the studies that have been made heretofore on the nutritive value of the proteins of oilseeds have been made on the seeds themselves, on products prepared for animal feeding or on specially prepared fractions. There are now available flours made from soybeans, peanuts, and cottonseed specially prepared for human use. It seemed desirable, therefore, to investigate the nutritive value of these products which may be used extensively in our dietaries and to determine how effectively their proteins supplement the proteins of wheat flour. There is at present no absolute value that can be assigned to the nutritive value of a protein and it is, therefore, necessary to make a comparison with products such as milk and casein, which have been studied extensively and are known to have high nutritive value.

#### EXPERIMENTAL

The products which served as sources of protein in these studies were as follows: A commercially prepared casein, spray-process skim milk powder containing 37.2% protein and 0.8% fat, a blend of two varieties of soft wheat containing 12.42% protein, commercially milled flour from the same wheat containing 10.60% protein, commercial soybean, peanut, and cottonseed flours made by the expeller process. These products contained, respectively, 49.68, 50.94, and 45.05% protein and 5.72, 8.11, and 15.4% fat.

All diets contained 2% cod liver oil and 4% salt mixture (Osborne and Mendel, '19). The remainder of each of the diets consisted of a combination of either one or more of the protein sources enumerated above together with sufficient corn oil to adjust the total fat content of the diet to 8%, and sufficient dextrinized cornstarch to adjust the protein content to either 9.1 or 15%.<sup>2</sup> The dextrinized cornstarch carried a vitamin mixture which provided in each 100 gm. of diet

<sup>2</sup> The following conversion factors were used in calculating the amounts of protein in the materials from their nitrogen content: wheat flour, 5.70; whole wheat, 5.83; peanut, 5.46; soybean, 5.71; cottonseed, 5.30; casein and lactalbumin, 6.38; PCS, 5.49 (Jones, '31).

thiamine hydrochloride 0.2 mg., pyridoxine hydrochloride 0.2 mg., riboflavin 0.3 mg., calcium pantothenate 0.3 mg., niacin 1 mg., and choline hydrochloride 10 mg. These vitamins were added to a portion of the dextrinized constarch in an aqueous solution and the product was dried at 50°C.

The data presented represent results obtained with thirty-one lots of albino rats comparable in all respects. Each lot consisted of twelve animals equally divided with respect to sex and having initial weights of 55 to 60 gm. They were housed in individual cages having wide mesh screen bottoms kept in rooms maintained at 76°F. ( $\pm 3^\circ\text{F.}$ ) and with about 55% relative humidity. The animals were weighed twice weekly and the diets were fed ad libitum. Enough of the stock flour mixtures were prepared to last throughout the 6-week feeding periods. Fresh diets were prepared weekly, usually in kilogram quantities, by incorporating into the stock flour mixtures the other non-protein dietary ingredients.

The first series of experiments was designed to show the growth-promoting values of the proteins of the products selected. In view of possible supplementary effects between the proteins of the three oilseeds a mixture (PCS) of equal parts of peanut, cottonseed, and soybean flours was also fed. The low protein level of 9.1% was used for comparative purposes because it was the maximum that could have been introduced into the diet by the wheat flour and still leave room for the non-protein ingredients.

A second series of experiments was conducted to ascertain the protein supplementary effect of adding the oilseed flours to wheat flour. For this purpose, sixteen lots of rats were fed diets containing mixtures of the supplements and wheat flour in the proportions of 5, 10, and 15 parts to 95, 90, and 85 parts, respectively.

Besides the three oilseed flours used separately, a mixture (PCS) consisting of equal parts of the oilseed flours was similarly used as a supplement to the wheat flour. Skim milk powder was also included for comparison.

## RESULTS AND DISCUSSION

### *Comparative growth-promoting values*

The proteins of wheat flour were found to have comparatively low nutritive value (table 1). This is in accord with results previously obtained by other workers and with what is known regarding the amino acid deficiencies of wheat endosperm proteins. The proteins of

whole wheat promoted weight gains nearly twice those obtained with the patent flour.

At the same protein level (9.1%) the animals fed the soybean and cottonseed diets gained approximately  $4\frac{1}{2}$  times as much as those fed the wheat flour diet. The somewhat lower gains obtained with peanut flour amounted, however, to 4 times those supported by wheat flour.

TABLE 1

*Comparative growth-promoting values of the proteins of peanut flour, cottonseed flour, soybean flour, patent wheat flour, whole wheat, skim milk powder, and casein.  
(Duration of feeding tests — 42 days).*

SOURCE OF PROTEIN	9.1% PROTEIN IN DIET				15% PROTEIN IN DIET			
	Lot no.	Average weight gain	Average gain per gm. protein consumed	Average food consumed	Lot no.	Average weight gain	Average gain per gm. protein consumed	Average food consumed
		gm.	gm.	gm.		gm.	gm.	gm.
Patent wheat flour	268	19 (10-30)	0.75	278	...	.....	...	...
Whole wheat flour	269	36 (30-45)	1.15	342	...	.....	...	...
Peanut flour	271	75 (57-121)	1.95	419	302	158 (97-222)	1.81	584
Cottonseed flour	270	85 (70-97)	2.05	455	303	138 (92-215)	1.67	552
Soybean flour	288	87 (51-115)	2.35	408	304	137 (70-190)	1.71	537
P.C.S. <sup>1</sup>	298	84 (63-177)	2.22	415	312	145 (87-186)	1.81	532
Skim milk powder	307	141 (97-193)	2.78	560	308			
					316	155 (97-208)	1.95	528
Casein	295	100 (90-111)	2.34	470	305	173 (123-230)	1.99	579

<sup>1</sup> Mixture of equal parts of peanut, cottonseed, and soybean flours. The figures in parentheses indicate the ranges of weight increases.

The results obtained with the mixture (PCS) of the three oilseed flours when compared with each one separately do not clearly indicate supplementary relationships among them. This mixture supplied protein having about the same growth value as that of soybean or cottonseed flour, but considerably higher than that of peanut flour.

At the 9.1% protein level skim milk powder and casein proved superior to the oilseed flours either on the basis of weight increase of the animals or on the gain in weight per gram of protein consumed.

Table 1 also shows the results obtained when the protein materials (with the exception of the wheat flour and whole ground wheat) were similarly fed at a 15% protein level in the diet. A comparison of the weight gains obtained when the diets supplied 15% protein with those obtained at the lower level brings out some interesting contrasts. With

9% protein in the diet, the average gain in weight of the animals fed peanut flour was 75 gm., the lowest obtained with the oilseed flours. At the higher protein level the corresponding weight gain was 158 gm., amounting to an increase of over 100% of that obtained at the 9% level and which placed peanut protein in the lead of the other oilseeds. Soybean flour at 9% protein level with a weight gain value of 87 gm. showed a higher value than peanut flour, but at the 15% level the reverse was true.

Quite different results were obtained with the milk proteins when fed at the two levels. With skim milk powder at a 9% protein level the animals gained 141 gm. However, when fed at a 15% protein level, the increase in weight-gain was only about 6% above that obtained at the lower level and not much higher than that obtained with peanut flour at the same protein level. Casein, on the other hand, proved to be much more efficient at 15% than at a 9% level.

The high value found for skim milk powder when fed at the lower protein level in the diet indicates its superior nutritional quality, and that at the lower level it was supplying almost enough of all the essential amino acids to meet the growth requirements of the young animals, since there was not much increase in weight gain when the protein level was increased to 15%. Casein, on the other hand, showed a much greater growth value at the higher than at the lower levels. It is now known through numerous studies that casein is deficient in cystine, an observation first made by Osborne and Mendel ('15) nearly 30 years ago.

#### *Proteins of the oilseeds and milk as supplements to wheat flour proteins*

The data given in table 2 show that the comparative effectiveness of the different supplements tested here varies with the proportions in which they are added to the wheat flour.

When mixed with wheat flour in the proportions of 5 to 95 parts soybean flour has a higher supplementary value than peanut flour or cottonseed flour, and has about the same value as that of the mixture of the three oilseeds (PCS), but is inferior to that of skim milk powder. It is of interest to note that soybean flour has about the same value as that of ground wheat alone at the same protein level in the diet (table 1), and twice the value of wheat flour alone.

In a mixture consisting of 10 parts of the supplement and 90 parts of wheat flour, soybean flour showed practically the same supple-

mentary value as skim milk powder, and produced weight increases 4 times that of the wheat flour alone.

A mixture of 15 parts of soybean flour and 85 parts of wheat flour showed a growth-promoting value exceeding that obtained with a mixture of skim milk powder and wheat flour in the same proportions (15:85). These results indicate that the most economical use of skim milk powder as a supplement to wheat flour can be made when it is mixed with the latter in a proportion not much greater than 10%. Although a somewhat higher value was obtained with 15 parts in the mixture, the point of diminishing return apparently begins when the proportion of skim milk powder to wheat flour is not far from 10 to 90 parts.

TABLE 2

*Growth-promoting values of the proteins of soybean flour, peanut flour, cottonseed flour, and skim milk powder as supplements to the proteins of patent wheat flour. (All the diets contained 9.1% protein.)  
(Duration of feeding tests—42 days.)*

	Lot no.	Average weight gain	Average gain per gm. protein consumed	Lot no.	Average weight gain	Average gain per gm. protein consumed	Lot no.	Average weight gain	Average gain per gm. protein consumed
		gm.	gm.		gm.	gm.		gm.	gm.
Wheat flour alone	268	19 (10-30)	0.75	...	...	...	...	...	...
Supplements		Supplement — 5			Supplement — 10			Supplement — 15	
Peanut flour	272	29 (20-38)	0.99	273	44 (32-55)	1.32	274	48 (36-69)	1.57
Cottonseed flour	275	24 (9-41)	0.91	276	41 (30-53)	1.29	277	42 (29-52)	1.30
Soybean flour	289	39 (26-52)	1.38	290	75 (57-96)	2.16	291	93 (77-114)	2.27
P.C.S.									
mixture <sup>1</sup>	299	36 (28-47)	1.16	300	48 (36-70)	1.40	301	70 (53-85)	1.80
Skim milk powder	319	49 (36-60)	1.44	320	77 (60-89)	1.86	321	85 (67-106)	2.06

<sup>1</sup> A mixture of equal parts of peanut, cottonseed, and soybean flours. The figures in parentheses indicate the ranges of wheat increases.

The data given in table 2 show the supplementary protein values of equal amounts of the oilseed flours and skim milk powder when mixed with wheat flour. Because of the differences in protein content of the supplementing materials fed, the figures do not strictly represent values for equal amounts of protein, but rather for equal amounts of the materials. For instance, in the diet containing a mixture of 5 parts of peanut flour and 95 parts of wheat flour, the peanut flour contributed 1.8% of protein and the wheat flour 7.3%. On the other hand, the same

amount of skim milk powder similarly used, supplied 1.4% protein and the wheat flour supplied 7.7%. It is believed that for practical purposes the comparative values will prove more useful when based on the amounts of the supplements used than on the amounts of the proteins supplied by the supplements.

Mixtures produced by addition of as little as 5 parts of cottonseed, peanut or soybean flour to 95 parts of wheat flour contained 16 to 19% more protein than the wheat flour alone and had a very definitely greater growth-promoting value of the protein.

Because of their high content of good quality protein, soybeans, peanuts, and cottonseed offer an economical means of supplying dietary protein especially in times of scarcity of protein of animal sources. In some respects these plant proteins compare favorably with milk proteins, which are generally regarded as among the best. They are well adapted to enhance the nutritional value of the proteins of wheat by supplementation.

An attempt to correlate with their amino acid composition either the growth-promoting values of the oilseeds or their values as supplements to the proteins of wheat flour is not very satisfactory because there are not sufficiently adequate data available on the amino acid content of protein foods. Most of the data available on amino acid composition were obtained on isolated proteins. Consequently, the values do not represent the foods as a whole from which the proteins were isolated. Furthermore, these values for the isolated proteins are too low in many cases because of unavoidable losses involved in their recovery from the protein hydrolysates. Methods for determining amino acids in foods without isolating the proteins are largely in the tentative stage.

It is generally recognized, however, that lysine is an outstanding amino acid deficiency in wheat flour. Available data leave little doubt that the proteins of the oilseed flours contain sufficient lysine to compensate for its deficiency in wheat flour. It also appears that wheat flour is low in threonine and valine as compared with soybean and cottonseed flours. Arachin, the preponderant protein in peanuts, is well known to be deficient in methionine (Beach and White, '37), although the total proteins of peanuts supply a satisfactory amount of this amino acid. Compared with wheat flour, soybeans and cottonseed are low in methionine. Almquist, Mecchi, Kratzer, and Grau ('42) showed by feeding experiments with chicks that the principal growth-limiting deficiency in raw soybean protein is that of methionine, and that heated soybean protein is slightly deficient in this amino acid for

the chick at a 20% protein level in the diet. Hayward and Hafner ('41) also found that for rats and chicks the proteins of raw soybeans were deficient in available methionine as well as cystine.

#### SUMMARY

The growth-promoting values of the proteins of soybean, peanut, and cottonseed flours were compared by the rat-growth method, and also their values as supplements to the proteins of wheat flour.

Soybean, peanut, and cottonseed flours contain proteins of high nutritive value and offer an excellent means of supplying dietary protein to extend and partially replace protein foods of animal origin.

These plant proteins are well adapted to enhance the nutritive value of the proteins of wheat flour. The addition of as little as 5 parts of peanut, soybean or cottonseed flour to 95 parts of wheat flour produced mixtures containing 16 to 19% more protein than wheat flour alone and a protein combination that was definitely superior in its growth-promoting value to the same quantity of protein from wheat flour.

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# THE UTILIZATION OF THIAMINE IN THE HUMAN SUBJECT: THE EFFECT OF HIGH INTAKE OF CARBO- HYDRATE OR OF FAT<sup>1</sup>

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## THREE FIGURES

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That the requirement for the B vitamins in the animal depends in part upon the composition of the diet with respect to carbohydrate and fat was first suggested by Funk ('14). Later, Evans and Lepkovsky ('29) found the rate of growth of rats fed diets high in fat increased when compared with those receiving diets high in carbohydrate. This they attributed to a "sparing action" of fat. Since that time considerable evidence has accumulated to indicate that in animals the requirement for thiamine is decreased as the fat content of the diet is increased (Williams and Spies, '39; Stirn, Arnold and Elvehjem, '39; Banerji, '40; Arnold and Elvehjem, '39). Some investigators, however, have failed to confirm such a relationship and the existence of a "sparing action" of fat has been questioned (Gregory and Drummond, '32; Kemmerer and Steenboek, '33; Sure and Buchanan, '35).

In man, studies to clarify this relationship have been few in number and the results to date equivocal. Widenbauer and Wieland ('39) observed increased utilization of thiamine following the addition of extra carbohydrate to the diet but this they attributed to the concomitant increase in total caloric intake. Schroeder and Benacchio ('39) and Wang and Yudkin ('40), however, found the output of thiamine in the urine reduced when the proportion of carbohydrate in the diet was increased without alteration in caloric intake. On the other hand no difference in urinary thiamine excretion during alternate periods of high fat and high carbohydrate intake could be detected by Cahill ('41). Thus, since it is important for both theoretical and practical reasons to know whether the type of food ingested alters in any

<sup>1</sup> Sponsored by a fund provided by Mrs. Arthur W. Thompson.

viously under the same conditions (Elsom et al., '42) and represented a considerable increase in intake of thiamine over the previous diet for all the subjects. The purpose of the supplement was to raise the thiamine intake sufficiently to bring about a urinary concentration of the vitamin that would permit accurate determination with negligible interference by the urine blank. Thiamine in urine, feces and food was determined by the thiochrome method as described in a previous paper (Elsom et al., '42).

#### STATISTICAL METHODS

Comparison of means was made by the method described by Goulden ('39) for small samples using Fisher's Table of "t." Regression lines were calculated by the method of least squares. The significance of the regression coefficients also was tested according to Goulden. Values of *P* in all of the tables were evaluated by the usual standards, *P* of 0.05 or less being considered significant and of 0.01 or less, highly significant.

#### RESULTS

All of the six subjects showed a trend of rising thiamine excretion during the basal diet period. This was caused by the increased intake of thiamine coincident with the beginning of the experiment as described above. Unpublished observations by the authors have shown that appreciably increasing the daily thiamine intake of man is followed by a stepwise increase in daily excretion in the urine. This increase in excretion occurs at a fairly uniform rate for each individual.<sup>4</sup> The response to altered thiamine requirement on different diets under these conditions is manifested mainly by changes in the regression of excretion with time and is not necessarily detectable by comparison of the mean daily output. This was found true in the present experiment. Thus, increasing the carbohydrate content of the diet over that of the basal period was followed in five of the six subjects (1 to 5 in figures 1, 2, 3) by decreased excretion of thiamine.<sup>5</sup> In subjects 2, 3, 4 and 5 this took the form of interruption or reversal of the rising trend of thiamine excretion (figs. 1, 2 and 3 and table 2); in subject 1 it was manifested by a lowered mean daily output (table 3). The mean excretion in subjects 2 and 3 showed a statistically significant rise during both the high carbohydrate and high fat periods.

<sup>4</sup> Although when plotted against time the increase is not rectilinear, it may be considered so over short periods without introducing appreciable error.

<sup>5</sup> The decrease was statistically significant in subjects 1, 3 and 4, and fell short of statistical significance in subjects 2 and 5.

This is explained by the recent rise in their thiamine intake and, in view of the reversal in the rising trend of excretion during these periods, has no bearing on the effects of the diet studied. Subject 6 showed no response. The alteration in thiamine excretion is well shown in the graphs for subjects 1 to 6, and the significance of the changes can be evaluated from the statistics in tables 2 and 3. It should be noted that the change from basal to high carbohydrate diet resulted in the same alteration of thiamine excretion regardless of whether a period

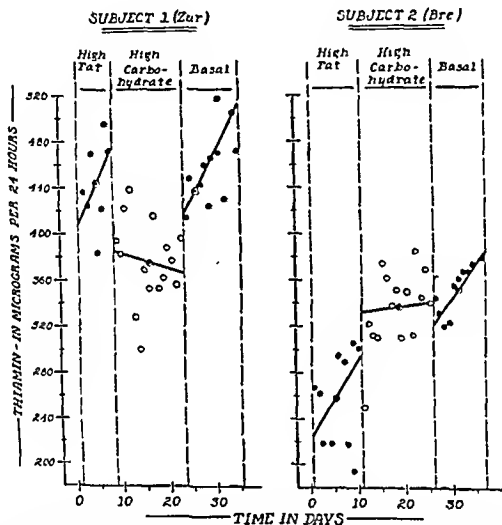


Fig. 1 Diminished excretion of thiamine during high carbohydrate diet period shown in subject 1 by decrease in mean output and in both subjects by change in slope of regression lines. Each point represents excretion of thiamine in urine during a 24-hour period.

of high fat intake intervened. It is apparent also that following the high carbohydrate diet the regression lines in the subsequent periods, whether basal or high fat, resume at a lower point than would have been the case had the high carbohydrate diet not intervened. Thus, the thiamine supply apparently had been depleted to such an extent that the accumulated "stores" were largely consumed.

The mean daily excretion of thiamine during the high fat period was increased over that of the basal period by a significant amount in

subjects 1, 2, 3, 4 and 6. However, this increase is adequately explained by the effects of the thiamine supplement as already mentioned and cannot be considered of importance since, with the exception of subject 6, the slopes of the plotted values did not change appreciably at this time. Since the "thiamine-sparing action" of fat, if any, should be

TABLE 2

*Effect of composition of diet on thiamine excretion in urine: comparison of regression.*

SUBJECT	BASAL DIET (A)			HIGH FAT DIET (B)			HIGH CARBOHYDRATE DIET (C)		
	Y	$S_b$	P	Y	$S_b$	P	Y	$S_b$	P
1	$Y = 7.25x + 415$	7.16	0.30	$Y = 7.73x + 417$	3.30	>0.5	$Y = 1.15x + 385$	2.25	0.08
2	$Y = 5.80x + 225$	1.39	<0.01	$Y = 5.78x + 319$	1.17	<0.01	$Y = 2.20x + 325$	1.62	0.2
3	$Y = 17x + 234$	5.70	0.02	$Y = 8.6x + 317$	3.75	0.05	$Y = 3.8x + 406$	0.82	<0.01
4	$Y = 1.26x + 96$	0.47	<0.02	$Y = 1.54x + 111$	0.79	0.075	$Y = 3.7x + 107$	1.56	<0.05
5	$Y = 1.89x + 69$	0.5	<0.01	.. .. .	....	....	$Y = 0.26x + 85$	0.47	0.06
6	$Y = 75x + 205$	1.57	0.28	$Y = 4.15x + 222$	2.62	0.15	$Y = 1.94x + 214$	0.98	0.07

\* Significance of differences of regression coefficients: (A) — (B) = non-significant; (A) — C =  $P < 0.01$  for subjects 3, 4, 5, others non-significant; (C) — (B) =  $< 0.01$  for subject 3,  $< 0.02$  for subject 4,  $< 0.05$  for subject 1, others non-significant.

TABLE 3

*Effect of the composition of the diet on thiamine excretion in urine: Comparison of means.*

SUBJECT	THIAMINE EXCRETION IN MICROGRAMS PER 24 HOURS (MEAN AND STANDARD DEVIATIONS) DURING PERIOD ON			SIGNIFICANCE (P) OF THE DIFFERENCES BETWEEN MEANS		
	Basal diet	High fat diet	High carbohy- drate diet	Basal-high fat	Basal-high carbohydrate	High carbohy- drate-high fat
1	444 ± 38	467 ± 47	376 ± 36	0.10	0.02	<0.01
2	257 ± 41	351 ± 29	341 ± 34	<0.01	<0.01	0.8
3	327 ± 70	381 ± 60	377 ± 38	<0.01	<0.01	0.3
4	107 ± 13	123 ± 16	124 ± 24	0.02	0.20	0.7
5	83 ± 11	.....	85 ± 9	.....	0.27	.....
6	217 ± 27	247 ± 30	230 ± 95	0.05	0.15	0.9

demonstrated in these subjects by differences in the excretion of thiamine between the basal and high fat periods, where the carbohydrate intake is not strikingly altered, and since no differences were observed, no "sparing action" is indicated.

In subject 6 there is a perceptible increase in slope during the high fat period as compared with the basal period which might be construed as evidence that fat was decreasing the need for thiamine with resultant greater surplus for excretion. The difference, however, fell short of statistical significance by a considerable margin. Furthermore, this subject showed a mean increase in the urine of 1.3 gm. N per 24

hours on the high fat diet at the same time that there was a loss of 0.7 kg. in body weight. The increase in urinary nitrogen, together with the weight loss, suggests a failure to use efficiently the high proportion of fats supplied by the diet. Increased output of thiamine in these circumstances might be explained in part by thiamine released through destruction of tissues and in part by the lower level of food utilization.

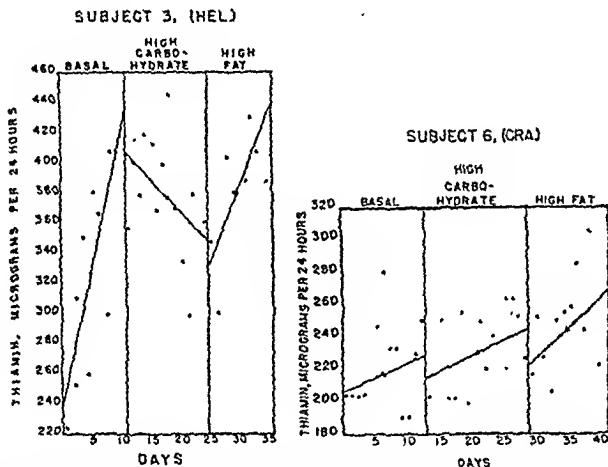


Fig. 2 Subject 3: rising excretion of thiamine during basal diet period interrupted by change to high carbohydrate diet with resultant decrease in excretion. Resumption of rise during subsequent high fat diet period. Subject 6: no significant effect of diet.

Nitrogen excretion, determined in two other subjects (4 and 5) showed no change.<sup>6</sup> Body weight, likewise, was unchanged in subjects 1, 4 and 5. Subject 2 gained weight during the basal diet period (0.7 kg.), probably due to the high calorie intake. The upward trend in weight was interrupted during the high carbohydrate period and resumed again when the high fat regime was instituted. A total of 1.3 kg. was gained at this time. Since subject 3 was on a similar regime, it is probable that similar changes in weight occurred.

No differences were observed either in free or total thiamine in the feces of two subjects in any of the experimental periods (table 4.)

<sup>6</sup>We are indebted to Dr. Hellen Goudsmit for all determinations of urinary nitrogen.

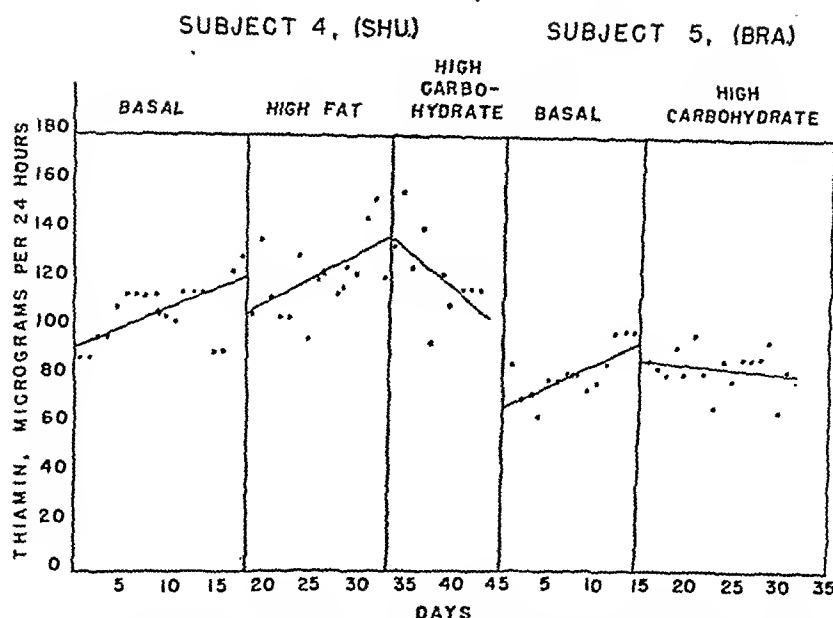


Fig. 3 Subject 4: absence of effect on thiamine excretion during high fat diet period contrasted with decrease during high carbohydrate diet period. Subject 5: rising trend of thiamine excretion during basal diet period reversed during high carbohydrate diet period.

TABLE 4

*Excretion of thiamine in feces during the different experimental periods: Comparison of means.*

SUBJECT	MEAN THIAMINE EXCRETION MICROGRAMS PER 24 HOURS					
	Basal diet		High fat diet		High carbohydrate diet	
	Free	Total	Free	Total	Free	Total
1	121 ± 79 <sup>1</sup>	558 ± 279	57 ± 33	374 ± 156	63 ± 20	600 ± 233
3	87 ± 66	299 ± 201	95 ± 77	263 ± 244	66 ± 30	202 ± 100

<sup>1</sup> Standard deviation.

## DISCUSSION

The output of thiamine in the urine is determined primarily by the amount of thiamine ingested (Elsom et al., '42). When the intake is maintained constant and other factors, such as exercise and infection, which have been found to influence its output, are eliminated as variables, the amount in the urine represents that portion of the intake which has not been (1) lost through the gastro-intestinal tract, (2) retained in the tissues or (3) used in metabolic processes.

At the levels of thiamine intake employed in these experiments all of the vitamin ingested is apparently absorbed from the gastro-intestinal tract and the thiamine in the feces may be accounted for by the

synthetic action of intestinal microorganisms. At higher levels of intake an increasing proportion of the ingested dose may be recovered from the feces (Elsom et al., unpublished observations). The lack of importance of the fecal thiamine in these experiments was established by the failure to obtain significant differences, either in free or total thiamine in the feces, in any of the three experimental periods.

Variations in the rate of absorption of thiamine from the digestive tract appear to explain to some extent daily variations in the urinary excretion of the vitamin (Elsom et al., '42) when the intake is maintained at a given level. However, factors causing variation in absorption presumably would be comparable in the different periods and could not explain the differences observed in these studies. Our subjects showed no indication of abnormal function of the digestive tract and it is unlikely that fermentative formation or destruction, varying in extent with the different diets, could have brought about the differences observed.

Cleavage of thiamine is known to occur in the course of its metabolic activity (Schultz, Atkin, and Frey, '40; Pollack, Ellenberg, and Dolger, '41; Gorham, Abels, Robins and Rhoads, '42). Although it is possible that the cocarboxylase concentration of tissues may increase as a result of the increased need for this coenzyme, animal experiments have failed to show that tissue thiamine is altered by high fat or high carbohydrate intake (Kemmerer and Steenbock, '33; Westenbrink, '34). Thus it seems unlikely that the decreased excretion observed during the high carbohydrate period could have been the result of retention in the tissues.

In view of these considerations the decreased excretion of thiamine during high carbohydrate intake in these subjects may be interpreted as indicating increased need for thiamine in the metabolism of the extra carbohydrate with consequent increase in the rate of its destruction.

The conflicting results of previous studies of the effect of fat on the thiamine requirement of animals appear to be explained by marked changes in the carbohydrate content of the diet when the effects of alteration in fat were being sought. Thus, a "sparing action" of fat was observed only when the fat was fed in conjunction with low carbohydrate intake (Evans and Lepkovsky, '29; Westenbrink, '34; Arnold and Elvehjem, '39; Salmon and Goodman, '37; Stirn et al., '39; Banerji, '40; Banerji and Yudkin, '42). When, on the other hand, diets high in fat were compared with diets of which the carbohydrate content



was not strikingly different, no "sparing action" was detected (Gregory and Drummond, '32; Kemmerer and Steenbock, '33; Sure and Buchanan, '35). The results of our experiments also show no effect of fat as such but a decided effect of carbohydrate.

The failure of Cahill ('41) to find that high carbohydrate intake altered thiamine excretion is more difficult to explain since the dietary conditions of this experiment were well controlled. One outstanding difference in his experiments from those here described was that his subjects were active, whereas ours were sedentary and their activity did not vary from day to day. Exercise is known to increase the need for thiamine (Guerrant and Dutcher, '40) and unless the amount of activity is regulated, would introduce a complicating factor. The use of single 24-hour specimens for comparison as in Cahill's experiments likewise would diminish the likelihood of finding differences because of the exaggerated influence of random variations in urinary output of thiamine in such a short collection period.

Although Widenbauer and Wieland ('39) regarded the decrease in excretion of thiamine in urine that occurred during a 5-day period of high sucrose intake as insignificant, statistical analysis of their data shows that, on the contrary, the decrease is statistically significant and that if the first day of each period is omitted the differences become highly significant. Their belief that the rise in total caloric intake was responsible for the difference that occurred is not supported by comparable experiments in which fat alone was increased, nor is it in agreement with our observations (Elsom et al., '42).

Thus it seems possible to reconcile the apparently conflicting results hitherto obtained. In view of the dependence of thiamine excretion in urine upon thiamine utilization when intake and other factors are kept constant, the decreased thiamine excretion observed when diets high in carbohydrate were fed indicates that the proportion of carbohydrate in the diet is an important factor determining the daily requirement of thiamine.

#### SUMMARY

1. The urinary excretion of thiamine was decreased in five out of six women when the carbohydrate-fat ratio in the diet was increased, all other dietary and environmental factors remaining constant. The decrease in thiamine excretion was manifested by interruption of a rising trend of excretion in four subjects and by a decrease in mean excretion in one.

2. No evidence of a "thiamine-sparing action" of fat was observed.

3. Excretion of free and total thiamine in the feces was not significantly affected by changes in carbohydrate-fat ratio of the diet.

4. The present observations demonstrate that the amount of carbohydrate in the diet is an important factor in determining the daily human requirement for thiamine.

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# THE PRODUCTION OF VITAMINS IN GERMINATED PEAS, SOYBEANS, AND OTHER BEANS

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The problem of attaining adequate nutrition for an Army, under difficult conditions of supply, with the subsequent prevention of nutritional deficiency symptoms, is an important one. In particular, the provision in desirable quantities of certain of the B-complex vitamins and ascorbic acid poses a difficult problem if available foods should include only canned meats and grain products as primary sources of the former, and canned or dehydrated vegetables for the latter. It is true that the commercial enrichment of grain products and commercial synthesis of ascorbic acid have served to ameliorate the situation somewhat. Nevertheless, the provision of any accessory source of these vitamins is highly desirable, and particularly so when a food group such as dried legumes provides a means of supplying them. This is true because dried legumes in addition to being plentiful and inexpensive, are also relatively light in weight and small in bulk in the quantities needed for 100 men. Furthermore, they are foods of excellent nutritive value in themselves, containing high quality protein, iron, calcium and certain vitamins.

As early as 1912, Hölst and Frölich proved experimentally that vegetables of high antiscorbutic potency became useless for the prevention of scurvy when dried and made suitable for military supply. Fürst, in 1912, proposed the use of germinated seeds as a solution to the problem. The menace of scurvy to British and Indian troops in Mesopotamia during 1915 and 1916 focused the attention of the Lister Institute group on the earlier work, with the result that Chick and Delf ('19) made an extensive investigation of the problem and found dry peas and lentils eminently suited for transport and production of considerable "antiscorbutic stuff", when germinated for use as required.

Using rat growth methods, Rose and Phipard ('37) compared the vitamin B and G values of fresh, frozen, dry, and sprouted peas and lima beans. They noted a loss of vitamin B and a gain of vitamin G (riboflavin) in the 14-day-old seedlings over that in the dry seed.

Burkholder and McVeigh ('42) found significant increases in the concentration of riboflavin, niacin, biotin, and pyridoxine during the germination of several kinds of seeds. They found little or no change in thiamine concentration following germination. All analyses were made on the plant material after drying at 70°C. for 12 hours.

In a subsequent paper, Burkholder ('43) summarized a comprehensive study of several of the B-vitamins in sprouted oats, wheat, barley, and corn. In all cases, sprouting increased the content of riboflavin and niacin, while the thiamine content remained unchanged. The results indicated considerable retention of the increased vitamins during dehydration of the sprouted seeds.

During the present war, the Australian Army, working through the Council for Scientific and Industrial Research and the facilities of the University of Adelaide ('42) have developed routine instructions for the proper sprouting of the "blue boiler" pea.

This laboratory has determined the productiveness of certain legumes during the process of germination with respect to thiamine, riboflavin, nicotinic acid, and ascorbic acid. On the basis of suitability for military use, three main types of legumes have been chosen; dried peas, soybeans, and other beans. The actual species under each type are as follows: Peas—Alaska, Wrinkled, Blackeye Cowpea; Soybeans—Peking, Bansei-1, Edsoy, Clemson, Higan-B, Easycook; Beans—Navy, Baby Lima, Red Kidney, Pinto.

It was desired to find out which type and species would be the most productive of the vitamins under consideration, and to discover possible effects of environmental variation.

#### EXPERIMENTAL PROCEDURE

##### *Method of sprouting legumes*

A quantity of each of the dry legumes (250 gm.) was weighed and placed in a sterilized burlap bag large enough to permit a five-fold increase in volume during soaking and sprouting. (In most cases duplicate samples of each legume were weighed and placed in separate bags.) The bags containing the legumes were immersed in tap water and allowed to soak for 8 hours. At the end of this period, considerable water had been absorbed and the legumes had increased in size and

softened considerably. (Excessive soaking, i.e., 24 hours, only served to accelerate the appearance of rot.) The bags were then suspended individually by cords attached to an overhead rack and allowed to drip into pans placed below. The rack was placed indoors about 3 feet from an open window which allowed free circulation of air. Following the initial soaking, the bags containing the legumes were thereafter immersed in fresh water for 20 minutes three times daily at 8-hour intervals.

Temperatures were taken twice daily with a thermometer suspended beside the bags, and occasionally an internal temperature was recorded. The latter practice was discontinued when it was found that the internal temperature ranged consistently from 2° to 10°F. cooler than the external (room) temperature, presumably due to the evaporation of water.

Each morning just before soaking, the legumes were emptied from the bags into clean dry beakers, weighed, and then weighed samples withdrawn for vitamin analysis. The remaining legumes were then returned to the bags for further sprouting.

### *Germination*

Some signs of germination were usually observed at the end of 24 hours, and in most cases sprouts were fully evident about 48 hours after the time that soaking was started. Germination counts made at that time on all varieties of legumes were as follows: Peas — Blackeye Cow Pea (96%), Alaska (95%), Wrinkled (96%); Soybeans — Peking (85%), Bansei-1 (97%), Edsoy (95%), Clemson (98%), Higan-B (100%), Easycook (100%); Beans — Baby Lima (81%), Red Kidney (0%) (no sprouts), Navy (35%), Pinto (84%).

### *Determination of moisture*

An example of the method used (in the field) for routine determination of moisture on the air-dry basis is as follows: 250 gm. of air-dry beans had increased in weight to 598 gm. at the end of 24 hours. The ratio  $250/598 = 0.42$  was applied to the weight of moist beans (50 gm.) taken out for analysis. This gave the weight of air-dry matter in the sample removed for analysis (21 gm.). This value was subtracted from the original 250 gm. to determine the calculated weight of legumes on the air-dry basis returned in the bag for further sprouting (229 gm.).

Results obtained from the actual determination of moisture (drying to constant weight in an oven at 100°–104°C., and allowing samples to

come into atmospheric equilibrium) were in close agreement with the calculations described above.

### *Ascorbic acid*

Determination of ascorbic acid was carried out by a modification of the method of Mindlin and Butler ('38), the modification being developed during the course of this investigation. It was found that the preliminary preparation of the samples, particularly soybeans, as carried out according to methods described in the literature, including that of Loeffler and Ponting ('42), did not provide for the effective removal of protein and other interfering substances. As a result, it was impossible to measure the degree of reduction of the indophenol dye because of the presence of a simultaneously appearing precipitate which had the effect of increasing turbidity, thus interfering with the passage of light through the solution to be measured.

The following method was finally developed and used with success: A weighed sample of sprouted legumes was finely comminuted and extracted in 2% metaphosphoric acid by means of a Waring blender, and then centrifuged. An aliquot of the supernatant liquid was withdrawn and treated for 15 minutes with  $\frac{1}{2}$  volume of 25% trichloroacetic acid to precipitate the interfering substances referred to above, and then centrifuged. An aliquot of this supernatant solution was placed in a photoelectric colorimeter, and a solution of buffered 2,6 dichlorophenol indophenol dye was added. After 30 seconds, the galvanometer deflection was read and the remaining dye color was destroyed with a few crystals of pure ascorbic acid before taking the second reading. After determining the deflection due to the dye solution alone, the ascorbic acid content of the unknown was calculated. Recoveries of 90-100% added ascorbic acid were obtained using this method (i.e. after removal of interfering substances by the use of trichloroacetic acid in the presence of metaphosphoric acid, as described).

### *Thiamine — riboflavin — nicotinic acid*

Thiamine was determined by the thiochrome method of Hennessy and Cerecedo as adopted by the Research Corporation Committee, riboflavin by the microbiological method of Snell and Strong ('39), and nicotinic acid by the microbiological method of Snell and Wright ('41).

### RESULTS AND DISCUSSION

The generation of the various water soluble vitamins investigated in this project is indicated in tables 1 and 2. It is apparent that sprouting

TABLE 1  
Vitamin content of sprouted legumes (mg. per 100 gm. moist legume).

	ASCORBIC ACID				THIAMINE			RIBOFLAVIN			NICOTINIC ACID			
	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	48 hrs.	72 hrs.	96 hrs.	48 hrs.	72 hrs.	96 hrs.	48 hrs.	72 hrs.	96 hrs.
Peas														
Alaska	5.8	16.7	25.9	23.1	26.3	0.23	0.24	0.22	...	0.32	0.52	1.28	1.14	1.14
Wrinkled	3.8	11.2	25.8	27.1	32.2	0.26	0.27	0.19	...	0.24	0.56	1.44	1.60	1.11
Black eye cowpea	4.0	11.0	26.4	30.8	31.4	...	...	...	...	...	...	1.28	Rot	Rot
Average	1.5	14.0	26.0	27.1	31.0	0.25	0.26	0.21	...	0.28	0.54	1.33	1.52	1.44
Soybeans														
Peking	2.0	8.1	14.4	11.3	11.2	0.27	0.33	0.23	0.56	0.46	0.35	1.04	1.00	0.93
Bansel-1	1.9	3.8	6.8	7.6	Rot	0.29	0.26	0.22	0.50	0.11	0.34	1.12	1.08	0.96
Edsoy	3.5	11.2	16.2	14.4	15.1	0.30	0.32	0.24	0.56	0.18	0.45	1.14	1.18	1.20
Clemson	3.0	7.2	12.7	11.2	13.0	0.36	0.33	0.25	0.44	0.47	0.41	1.10	1.21	1.15
Higan-B	3.0	7.8	11.6	11.1	Rot	0.31	0.26	0.23	0.52	0.16	0.40	0.99	1.04	1.09
Easycook	4.5	16.0	12.8	12.0	12.4	0.27	0.26	0.21	0.52	0.50	0.41	0.89	1.00	1.06
Average	3.2	9.0	13.5	12.0	12.9	0.29	0.29	0.23	0.53	0.16	0.40	1.05	1.09	1.06

Note: Each value represents the average analysis of 2-6 sproutings.



TABLE 2  
Vitamin content of sprouted legumes (mg. per 100 gm. air-dry matter).

	ASCORBIC ACID						THIAMINE			RIBOFLAVIN				NICOTINIC ACID				
	0 hrs.	24 hrs.	48 hrs.	72 hrs.	96 hrs.	120 hrs.	0 hrs.	48 hrs.	72 hrs.	96 hrs.	0 hrs.	48 hrs.	72 hrs.	96 hrs.	0 hrs.	48 hrs.	72 hrs.	96 hrs.
Peas																		
Alaska	0	13.7	35.8	64.6	66.1	76.7	0.71	0.51	0.62	0.63	0.22	...	0.37	1.55	2.75	2.78	3.68	4.25
Wrinkled	0	8.2	27.2	71.8	82.9	102.8	0.84	0.62	0.76	0.62	0.38	...	...	1.81	3.15	3.42	4.48	4.65
Black eye																		
cowpea	0	8.8	33.7	70.4	82.2	100.0	0.84	...	...	...	0.31	...	...	...	2.40	3.18	Rot	Rot
Average	0	10.2	32.2	68.9	77.1	93.2	0.80	0.56	0.69	0.62	0.30	...	0.75	1.68	2.77	3.13	4.08	4.45
Soybeans																		
Peking	0	4.0	18.3	38.7	42.0	48.7	1.07	0.62	0.89	0.81	0.32	1.21	1.31	1.34	1.85	2.43	2.83	3.51
Bansai-1	0	4.2	9.0	17.1	21.3	Rot	0.98	0.70	0.67	0.61	0.55	1.34	1.02	1.02	2.40	2.78	2.74	2.58
Edsoy	0	8.1	27.6	45.3	52.2	62.3	1.01	0.73	0.84	0.82	0.80	1.35	1.36	1.53	1.50	2.82	3.33	3.06
Clemson	0	6.3	16.6	32.3	35.2	48.3	1.06	0.82	0.96	0.77	0.47	0.99	1.21	1.22	1.88	2.57	3.19	3.43
Higan-B	0	6.9	19.5	32.3	37.6	Rot	1.02	0.78	0.73	0.73	0.41	1.29	1.29	1.27	1.55	2.49	2.92	3.52
Easycook	0	10.4	28.1	37.0	45.5	52.7	0.89	0.69	0.75	0.74	0.43	1.31	1.49	1.51	1.45	2.31	3.00	3.88
Average	0	6.7	19.9	33.8	39.0	53.0	1.01	0.72	0.79	0.75	0.56	1.24	1.28	1.32	1.77	2.56	3.00	3.33

Note: Each value represents the average analysis of 2-6 sproutings.

causes a marked increase in ascorbic acid, a fair increase in riboflavin and nicotinic acid, and none in thiamine. This is over and above the vitamin content inherent to the non-germinated legume.

Generally speaking, the various varieties of peas were found to produce the greatest quantities of ascorbic acid, riboflavin and nicotinic acid. Soybeans, while less efficient in this regard, were nevertheless of considerable merit, and markedly superior to the other types of beans investigated, which showed a tendency to rot. The rotting, in turn, was associated with a cessation of vitamin production.

It was observed also that the rate of germination, for all types of legumes investigated, showed a direct correlation with the temperature of the room. In turn, the rate of generation of the vitamins (thiamine excepted) followed the same trend as the rate of germination.

A 100-gm. serving of sprouted peas and soybeans, before cooking, would provide approximately the following minimal quantities of nutrients, (mg.), according to the results obtained in this investigation. Ascorbic acid — peas (25.0), soybeans (12.0); riboflavin — peas (0.35), soybeans (0.45); nicotinic acid — peas (1.40), soybeans (1.00); thiamine — peas (0.24), soybeans (0.28). On the same basis, other beans would provide 3.0 mg. of ascorbic acid, 0.30 mg. of riboflavin, 0.30 mg. of nicotinic acid and 0.1 mg. of thiamine.

The values listed above are conservative average values obtained when the sprouts had attained 2 inches in length. In the summer months, this occurred after 72 hours, which included the soaking time. This period was found to be increased to approximately 120 hours at the lower temperature of the winter months. The 24-hour temperature range was as follows: summer, 68°-97°F.; winter, 64°-75°F. In addition, it was found that the sprouts became less tender and palatable when they exceeded two inches in length. When sprouted in the manner described and eaten at this stage, the peas and soybeans are very palatable, and possess an excellent flavor. It is considered that the final product would be highly acceptable as a palatable food as well as an accessory source of vitamins. The antiscorbutic value of such sprouted legumes has long been proven where the supply of fresh or canned fruits or vegetables is limited. It also appears that these legumes are of additional value because of small but significant quantities of other water soluble vitamins.

#### SUMMARY

Dried peas, soybeans, and other legumes have been investigated in relation to their generation of ascorbic acid, thiamine, riboflavin, and

nicotinic acid following germination. It has been determined that, generally speaking, such sprouted legumes are an excellent source of ascorbic acid, fair as a source of riboflavin and nicotinic acid, and poor for thiamine. The various varieties of peas were more productive of the vitamins concerned than were soybeans, which, however, were of considerable merit and markedly superior to the other types of beans investigated. A temperature effect was determined, indicating that both germination and the generation of vitamins proceeded at a faster rate at the higher temperature range. The applicability to military conditions of this method of producing an accessory supply of water soluble vitamins is indicated.

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two periods and 0.76, 0.77, and 0.76 in the fourth, fifth, and sixth periods. Analyses for periods 5, 7, and 8, respectively, of the last three experiments with water showed a complete absence of methane in the expiratory gases and in period 8 on October 25th only 0.012% of methane.

The effect on the R. Q. of the ingestion of 25 gm. of glucose was evident in three experiments by the third period and reached its maximum in the third and fourth periods in three experiments and in the sixth period in two experiments. In the two experiments in which base-line values were obtained, the maximum rises above the base-line were 0.11 and 0.07. The average quotients for the successive periods after ingestion of glucose were 0.73, 0.76, 0.80, 0.80, 0.79, 0.80, 0.79, and 0.78. The rises in the quotients of these goats in this series of experiments were nearly as large as those noted with man after the administration of 50, 62, or 100 gm. of glucose. It is assumed that these are true metabolic quotients, as an attempt was made to introduce the solution of glucose into the true stomach and thus avoid fermentation. Unfortunately the methane content of the chamber air was determined in only three periods in the glucose experiments (period 8 after glucose on October 27th and periods 4 and 7 on November 2nd). In these instances it was found to be 0.007, 0.003, and 0.024%, respectively. It is questionable whether the maximum amount is significant.

There are some indications that the metabolism of carbohydrates is different in ruminants from that in other mammals. Krzywanek and Brüggemann ('33) report that the administration of glucose into the true stomach of the sheep had no appreciable effect on the level of blood sugar. This absence of effect they ascribe to fermentation of the sugar in the stomach of the ruminant. Cutler ('34) found that the normal resting blood sugar of twenty-six goats ranged in the majority of cases from 40 to 50 mg. per 100 ml. and that the oral administration of 1.5 gm. of glucose per kilogram of body weight caused the blood sugar to rise from a base-line of 42 mg. to only 59 mg. She offered as an explanation that the goat is a ruminant and, therefore, possibly the rate of absorption is relatively slow. Magee ('32) found that the blood sugar of goats rose from a base-line of 80 mg. to 110 mg. per 100 ml. after 50 gm. of sucrose and 150 gm. of starch, when the animal had fasted 48 hours. After longer fasting, the rise was not so great.

The effect on the R. Q. of the ingestion of 25 gm. of fructose was evident promptly, as there was a definite rise above the base-line in the first and second periods after the dose. The maximum occurred in the third and fifth periods. The average quotients after fructose

were, by periods, 0.81, 0.82, 0.86, 0.84, 0.85, 0.84, 0.82, and 0.82. Significant amounts of methane were detectable in only two of the periods after fructose in which these analyses were made (October 24th, periods 3 and 8, 0.007 and 0.041%; October 28th, periods 4 and 8, 0.006 and 0.025%). The maximum rise was lower and occurred later with the goat than with man after administration of 50 or 100 gm. of fructose. The effect of fructose on the R. Q. of the goat resembles more nearly that found with cats (unpublished experiments) than that noted with man and with monkeys (Carpenter and Hartman, '44).

The effect of the ingestion of 25 gm. of galactose was evident in all four experiments by the fourth period. The results in the experiment on October 26th were irregular. It is possible that the solution might not have been introduced into the true stomach. However, the two analyses made for methane in this experiment indicate no fermentation. The last four periods in this experiment show a distinct rise in the R. Q. The maximum rises in the other three experiments were 0.08, 0.06, and 0.06 in the fifth, seventh, and fourth periods, respectively, after administration of the sugar. The average quotients after galactose were, by periods, 0.76, 0.75, 0.77, 0.77, 0.79, 0.79, 0.79, and 0.78. These resemble closely those after the ingestion of 25 gm. of glucose. Analyses for methane were made in a total of nine periods. In the several experiments, in the third period after ingestion of galactose, the methane values were 0.0, 0.015, 0.013, and 0.015%, respectively, and in the sixth or the seventh period they were 0.0, 0.031, 0.012, and 0.035%, respectively. In the eighth period on November 1st, one analysis showed 0.008%. In other words, in three experiments a measurable amount of methane was found in the third and the sixth or the seventh period after ingestion of galactose, but in the experiment in which it was doubted whether the solution was placed in the true stomach no methane was found. The finding of methane in several periods indicates that the metabolism of galactose in these goats was accompanied by fermentation. The effects of such amounts of methane on the R. Q. are negligible. The changes in the quotients of goats after galactose ingestion differ from those with man after 50 gm. of galactose, as the rise with man is much greater and the peak occurs earlier than with goats.

#### *Heat production and metabolism of carbohydrates*

The values for the heat production in the basal periods, calculated to the basis of 4 hours from the measurements of the respiratory exchange, and for the heat production during the 4 hours of measure-



ment after the ingestion of water and of the sugars are given in table 2. The values are not corrected for activity, as the purpose of the calculation of the heat production was to estimate the metabolism of the carbohydrates. Because of the variability in activity, no conclusions can be drawn as to the effects of ingestion of water or of the sugars on the output of energy. The metabolism of carbohydrates was calculated in the usual empirical manner from the measurements of the respiratory exchange, after correction for the metabolism of protein on the assumption that 15% of the oxygen absorption was ascribable to the oxidation of protein. The values are given in table 2.

TABLE 2

*Heat production and metabolism of carbohydrates before and after ingestion of water or sugar by goats.*  
(Values per 4 hours)

DOSE <sup>1</sup> AND DATE	GOAT NO.	SEX	BODY WEIGHT	HEAT PRODUCTION		CARBOHYDRATE METABOLIZED	
				Baseline	After dose	Baseline	After dose
- 1938			kg.	cal.	cal.	gm.	gm.
<i>Water</i>							
June 29	8	M	33.3	...	198	...	0.0
June 30	9	M	33.3	...	201	...	0.0
Oct. 25	2	M	37.0	...	177	...	3.6
Oct. 29	6	F	41.5	...	189	...	3.8
Nov. 4	5	F	45.1	...	207	...	6.5
<i>Glucose</i>							
Feb. 25	7	F	42.9	...	177	...	14.8 <sup>1</sup>
June 29	8	M	33.3	...	204	...	1.7 <sup>1</sup>
June 30	9	M	33.3	...	196	...	3.1 <sup>1</sup>
Oct. 27	4	F	39.2	179	173	3.5	13.2
Nov. 2	3	F	35.8	144	149	2.3	8.3
<i>Fructose</i>							
Oct. 24	2	M	39.2	197	201	10.6	19.8
Oct. 28	5	F	43.8	217	227	4.0	16.9
<i>Galactose</i>							
Oct. 26	3	F	35.4	186 <sup>2</sup>	165 <sup>3</sup>	3.0	4.2 <sup>2</sup>
Oct. 31	1	M	39.5	167	168	3.6	11.4
Nov. 1	2	M	36.7	189 <sup>2</sup>	168	0.0	3.8
Nov. 3	4	F	40.6	164	166	5.0	9.5

<sup>1</sup>In the experiments on Feb. 25, June 29, and June 30 the goat was given the solution to drink a few spoonfuls at a time; in all other experiments the solution was introduced by stomach tube.

<sup>2</sup>Goat active.

<sup>3</sup>Solution of galactose may not have been introduced into the true stomach.

The amounts of carbohydrate utilized in 4 hours in the base-line periods varied from 0 to 10.6 gm. The majority were under 4 gm. As no base-line values were determined in the experiments with water, no conclusion can be drawn regarding the effect of ingestion of water on the metabolism of carbohydrates. However, from the changes in the R. Q. in the experiments with water and from the general level of the base-line values for carbohydrate metabolized in the other series of experiments, there is evidence in three of the experiments that the metabolism of carbohydrates was slightly increased after the ingestion of 250 ml. of water. In the experiments with the sugars, the metabolism of carbohydrates was highest after the ingestion of fructose, both from the standpoint of absolute values and from the standpoint of increases above the base-line values. Even when the base-line value was high (10.6 gm. with a base-line R. Q. of 0.79), there was an increase of 9.2 gm. The carbohydrate metabolized after ingestion of glucose was the next in order of increase above the base-line, if the experiments in which the sugar was not given by stomach tube are disregarded. After ingestion of galactose the increases were the least, except in one experiment where the increase was 7.8 gm. With man the greatest increase is after fructose ingestion, whereas the increases after administration of the other two sugars are less and about the same. The rate of metabolism of carbohydrate after fructose ingestion is so high with goats that 25 gm. of this sugar might not suffice for much longer than 8 hours, whereas 25 gm. of galactose might supply the necessary carbohydrate for from 12 to 24 hours.

#### SUMMARY

The respiratory exchanges of four male and five female adult goats were determined 40 hours after withdrawal from food (1) under basal conditions and (2) in eight successive  $\frac{1}{2}$ -hour periods after the administration by stomach tube of 250 ml. of water at 37°–38°C., or of 25 gm. of glucose, fructose, or galactose dissolved in 125 ml. of water and an additional 125 ml. of water for rinsing.

Water produced a slight but somewhat delayed increase in the R. Q. Fructose caused the greatest increase in the R. Q. and the greatest increase in the metabolism of carbohydrates. Glucose was next in these effects, and galactose had the least effects. Qualitatively these results resemble much those found with man with these sugars. There was evidence of a slight amount of fermentation after the ingestion of galactose and of fructose by the goats.

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# THE ROLE OF DIETARY FAT AND LINOLEIC ACID IN THE LACTATION OF THE RAT

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In a long series of experiments summarized by Maynard and co-workers in 1941 it has been shown that dietary fat stimulates milk secretion in dairy cows. A somewhat greater quantity of milk was consistently produced on the higher fat diet. Maynard and Rasmussen ('42) used the growth rate of standardized litters to demonstrate that high-fat diets support better lactation performance in rats than low-fat diets. Corn oil and crude coconut oil seemed to have a similar effect despite their widely different fatty acid makeup.

In order to investigate possible explanations of the effect of fat on lactation performance experiments were set up to measure the growth of litters whose mothers were fed a diet free from fat as compared with varying amounts of corn oil, and hydrogenated fat. The composition of the diets is shown in table 1.

## METHODS

Mature female rats which had previously littered were mated and caged separately before parturition. After 12 but before 24 hours following parturition, the feed was removed and the litters standardized to six. Females were selected in pairs, triplets or quadruplets that were similar in body weight and in weight of litter for comparison of the diets. The females were fed equicaloric amounts of food usually corresponding to that consumed by the female on the fat-free diet. The young and mothers were weighed daily during a 17-day period to follow growth increases and to note individual characteristics of the litter. On the morning ending the 17-day period the young were etherized, the viscera removed and the carcasses frozen until chemical analyses were made. The results obtained with four females on each treatment are shown in table 2.

It is seen that when the diet contained 5.5, 11.3, or 19.2% of corn oil the growth of the litters was definitely superior to that on the low

fat diet. This suggested that an essential nutrient element was being supplied to satisfy metabolic needs at all levels of corn oil fed.

Comparisons were then made of the low-fat, corn oil and hydrogenated corn oil diets using trios of rats. The results (table 2) show the superiority of the corn oil over the low-fat diet. On the diet containing hydrogenated corn oil the young made less gain in weight than on the low-fat diet and the mothers lost more weight. These results cannot be given much reliance because balance studies revealed that as an average only 54.3% of the hydrogenated corn oil was absorbed, and, therefore, the calories available for maintenance and lactation were appreciably less than for the mothers fed the low-fat or corn oil diets.

Hydrogenated coconut oil was next selected for comparison because of its relatively low melting point and high digestibility (Hoagland and Snider, '43). In this comparison (table 2) less growth was obtained on the diet containing hydrogenated coconut oil than on the low-fat diet.

Carcass analyses of the young from these three studies showed that most of the extra gain in weight of the young on the corn oil diet was due to the deposition of extra body fat. There was only a slight increase in total protein deposited.

These data confirmed the earlier study from this laboratory in showing that corn oil in the diet of rats stimulates lactation performance. It appeared that something in corn oil, lost by hydrogenation, was responsible for the faster growth of the young rats. The possibility of a deficiency of the essential fatty acids presented itself.

#### *Linoleic acid not the first limiting factor*

To test the possibility that a deficiency of linoleic acid was retarding the growth of the young, two female rats with litters standardized to six were at parturition placed on a fat-free diet (described later). Each litter was divided into two groups of three. To one group of the young nursed by each mother was fed 1 drop of ethyl linolate per day. To the other group was given 1 drop of fully hydrogenated coconut oil per day. The feeding period was extended to 17 days. In neither case could any significant advantage be assessed to the group receiving the ethyl linolate.

On the basis of this preliminary test it was felt that any advantage due to ethyl linolate would result from a stimulus to the lactation process, and that poor growth of the young on the low-fat diet was not due to a deficiency of the essential fatty acids in the milk.

To test the lactation response due to linoleic acid and to obtain more data on the value of hydrogenated coconut oil four different nutrient comparisons were studied. They were, an ether extracted purified diet, the same supplemented with 125 mg. of ethyl linolate for each rat daily, a purified diet containing 10% hydrogenated coconut oil, and a purified diet containing 10% corn oil. The same low-fat diet shown in table 1 was used except that the corn starch was replaced with

TABLE 1  
*Composition of diets.*

INGREDIENT	LOW FAT	CORN OIL	CORN OIL	CORN OIL	HYDRO- GENATED CORN OIL, <sup>1</sup>	HYDRO- GENATED COCONUT OIL, <sup>2</sup>
	<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>parts</i>	<i>parts</i>
Casein	20	20	20	20	20	20
Yeast (extracted)	5	5	5	5	5	5
Corn starch	40	25	30	35	25	36.3
Sucrose	30	11.3	17	23.4	11.3	.
Bone meal (extracted)	2	2	2	2	2	2
Salts (Hawk and Osler)	3	3	3	3	3	3
Choline	0.1	0.1	0.1	0.1	0.1	0.1
Cystine	0.2	0.2	0.2	0.2	0.2	0.2
Fat	.	15	10	5	15	15
Total	100.3	81.6	87.3	93.7	81.6	81.5
Protein %	16.9	23.3	21.6	20.4	21.8	24.7
Fat %	0.2	19.2	11.3	5.5	17.3	18.5
Ash %	5.2	5.4	5.9	4.6	5.7	5.5

<sup>1</sup> The corn oil was hydrogenated in our laboratory to an I.V. of 30.

<sup>2</sup> The hydrogenated coconut oil (I.V. 0.6) was kindly furnished by Lever Brothers, New York, N. Y.

Each female received daily 40 I.U. of vitamin A, 4 I.U. of vitamin D, 0.2 mg. of alpha-tocopherol and 15 drops of rice bran concentrate, "Vitab". The females were irradiated for 30 minutes three times each week.

sucrose. In the high-fat diet 10 parts of the oil replaced an equicaloric amount of sucrose. The casein, yeast and bonemeal were extracted with ethyl ether continuously for 48 hours. The corn oil used in diet 3 was a purified grade with an iodine value of 126.3. The hydrogenated coconut oil used had an iodine value of 0.6. The Osborne and Mendel salt mixture was used. Generally a fat-free base was mixed. The appropriate amount of sucrose or fat to give the three diets was then added to it. The nutritive ratios of these diets were so adjusted that they would be the same when equal caloric intakes were fed. One gram



of the low-fat diet was fed to supply the same number of calories as 0.899 gm. of the high-fat diets. Analyses showed that the ether-extracted diet contained 18.2% of protein, 0.2% fat and 76.5% carbohydrates, the corn oil diet 22.0% of protein, 10.2% of fat and 61.2% carbohydrates.

The mother rats received supplements of the fat soluble vitamins dissolved in hydrogenated coconut oil. One drop of the supplements was given three times each week to supply 123  $\mu$ g. B-carotene, 969  $\mu$ g. 2 methyl 1-4 naphthoquinone, 21 mg. tocopherol, and 708 U.S.P. units of vitamin D weekly. Vitamin K was included in this supplement in view of the report of Harris and Mosher ('40) that rats may develop

TABLE 2

*The average growth and composition of litters of rats on an ether extracted diet and with added corn oil, hydrogenated corn oil or coconut oil.*

DIET	PERCENT FAT	CALORIC INTAKE	PROTEIN INTAKE	CHANGE IN WEIGHT OF MOTHERS	GAIN OF LITTER	CARCASS CONTENT OF	
						Protein	Fat <sup>1</sup>
			gm.	gm.	gm.	gm.	gm.
Ether extracted	0.2	1707	83.0	— 6	121.9	17.9	16.0
Corn oil	5.5	1752	93.6	+ 3	152.1	21.3	24.8
Corn oil	11.3	1762	94.0	— 8	161.2	20.7	29.0
Corn oil	19.2	1692	89.3	— 17	150.1	20.7	24.8
Ether extracted	0.2	1854	90.0	+ 3	131.7	20.1	17.6
Corn oil	19.2	1847	97.4	— 15	164.8	22.2	23.7
Hydrogenated corn oil	17.3	1845	98.0	— 25	114.3	17.7	13.1
Ether extracted	0.2	1546	74.5	— 11	158.0	24.1	22.0
Hydrogenated coconut oil	18.5	1536	86.3	— 8	134.0	20.9	19.0

<sup>1</sup> The dried carcasses were extracted with a mixture of anhydrous ethyl ether 50 parts and absolute ethyl alcohol 50 parts by volume.

a vitamin K deficiency when maintained on a diet containing an abnormal dietary fat constituent (dihydroxy-stearic acid) as its major fatty component. Two drops of a rice bran concentrate<sup>1</sup> were administered daily as a source of B-complex vitamins. Ethyl linolate was fed by dropper in 125 mg. daily dosages.

Mother rats were selected in quadruplets according to body weight, age and weight of litter, one for each dietary. In all, seven animals were fed equal caloric amounts of each diet and the weight change of mother and young recorded as formerly. The average nutrient intakes and weights of the four groups are shown in table 3.

<sup>1</sup> Vitab.

TABLE 3

*Nutrient intakes, live weights and weight gains of rats studied.*

DIET	TOTAL INTAKE	PROTEIN INTAKE	INITIAL WEIGHT		WEIGHT CHANGE OF MOTHERS	WEIGHT GAIN OF LITTER
			Mother	Litter		
	Cal.	gm.	gm.	gm.	gm.	gm.
Ether extracted	1253	59.8	247	35.6	— 13	110.6
Hydrogenated coconut oil	1220	63.2	257	35.0	— 21	107.9
Corn oil	1220	63.8	248	33.2	— 9	129.1
Ether extracted plus 125 mg. of ethyl linolate	1241	58.4	260	36.4	— 21	112.1
Least difference (odds of 1:100)	.	.	.	.	24	19.0
Significance of difference	.	.	ns	ns	ns	SS

The initial weights of the females and litters were quite similar. As an average, the mothers lost weight while suckling their young but no correlation could be shown between weight loss of the mothers and diets fed (or gain in weight of the litter). This differs from the observation of Maynard and Rasmussen ('42). They found that the mothers fed high fat diets lost more weight, or gained less, during lactation than those fed low-fat diets.

The gain in weight of those litters on the corn oil diet was decidedly greater than those of the other three diets.

It seemed important to measure the digestibility of the coconut oil since a low absorption would decrease the calories available and depress lactation performance. Studies were, therefore, carried out to compare the absorption of corn oil and hydrogenated oil when they constituted 10.2% of the same diets used in the lactation experiments. The average percentage of apparent digestibility determined with seven adult female rats was 92.3 for hydrogenated coconut oil and 94.5 for corn oil. It is clear that the differences in lactation response cannot be explained on the basis of absorption of the energy.

Carcass analyses of the litters reared on the different diets were made to determine the exact nature of the extra gain in body weight of the young suckled by mothers fed corn oil. The frozen carcasses were ground and dried in vacuo under CO<sub>2</sub> at 60°C. for 48 to 72 hours until constant weight was obtained.

For determination of the body fat a saponification method modified after that of Hurst ('33) was used. The ground, dried carcasses (about 50 gm.) were placed in a tall 600 ml. beaker and an equal weight of

KOH, 200 ml. EtOH, and 100 ml. of water were added. They were then heated on a hot plate just below boiling for  $1\frac{1}{2}$  to 2 hours, 50 ml. water were then added and the solution boiled for 30 minutes. The solution was filtered through glasswool to remove undissolved particles and washed with hot  $H_2O$ , making up to 300 ml. final volume.

The fat was determined using the Rose-Gottlieb wet extraction procedure. Fifteen ml. soap solution were acidified with 15 ml. 40% HCl and 15 ml. 95% EtOH added. Two extractions were made with 50 ml. portions of a mixture (1:1) of ethyl and petroleum ether. The fatty acids were dried under  $CO_2$  at  $95^\circ C$ . for 15 minutes and then weighed.

The iodine values for the extracted body fats and milk fats were determined by the A.O.A.C. method.

The results of the analyses for dry matter, fat, and iodine number are shown in table 4.

TABLE 4  
*Average carcass composition of the litters.*

DIET	FRESH WEIGHT	DRY MATTER	FAT	NON-FAT DRY MATTER	BODY FAT IODINE NO.
	gm.	gm.	gm.	gm.	gm.
Ether extracted	130.5	34.0	8.5	25.5	61.7
Coconut oil	128.5	33.4	8.4	25.5	57.3
Corn oil	147.5	41.5	13.4	28.1	80.4
Ether extracted with ethyl linolate	132.8	34.4	8.5	25.9	64.9
Least difference (odds of 1:100)	....	6.6	3.0	4.7	8.4
Significance of difference	..	SS	SS	ns	SS

The corn oil group contained more total dry matter and total fat than any of the other three groups. However, the non-fat dry matter in the carcasses of all groups was about the same, only a barely significant difference (odds of 1:20) in favor of the corn oil group existed. Practically all of the extra gain in weight of the corn oil group may be accounted for by increase in fat content of the carcasses.

The body fat iodine numbers show some interesting variations. As expected, the group on unsaturated corn oil showed a very significant increase in iodine number. The average iodine number of the group supplemented with ethyl linolate was significantly higher than that of the group on hydrogenated coconut oil. The iodine number of the body fats of the group receiving ethyl linolate was higher than that of the rats receiving only the ether extracted diet but this difference was not significant.

Milk fat and total solids were obtained from the stomach of the young rats killed immediately after they had suckled for a standardized period. The data were so variable that no relation could be shown between the diets fed and the amount of dry matter or fat secreted. The iodine numbers of the milk fats obtained on the respective diets were as follows: ether-extracted diet, 23.1; coconut oil diet, 26.6; corn oil diet, 37.8; ether-extracted diet with ethyl linolate 38.3.

The iodine numbers of the milk fat on the corn oil diet and on the extracted diet supplemented with 125 mg. of ethyl linolate per day were significantly higher than those on the other two diets (the least difference for odds of 1:20 was 11.7). The milk fat on the corn oil diet was always liquid and possessed a yellow-brown coloration while that on the extracted diet was rather clear and semi-solid or solid at room temperature. The litters from the corn oil diets had more oily skins and never developed a temporary scaliness which was sometimes observed in the other litters.

#### DISCUSSION

The data presented show that suckling rats make more rapid growth when their mothers are fed a purified diet containing corn oil than when fed a fat-free diet. This confirms the findings of Maynard and Rasmussen ('42). That all fats do not have equal values for lactation is shown by the fact that with the diet containing fully hydrogenated coconut oil the growth response was no better than with the fat-free diet. In the earlier study crude coconut oil appeared to be about equal to corn oil. This difference in response suggests that certain unsaturated fatty acids stimulate lactation performance. It appears clear that the more rapid growth of the young which were suckling mothers fed the corn oil diet is due either to the production of more milk or to an alteration in the quality of the milk in such a way that growth of the young is stimulated.

Feeding 125 mg. of ethyl linolate to each lactating female daily did not improve the growth of the young as compared with the ether extracted diet. Thus, this essential fatty acid does not appear to be a limiting factor in the growth of the suckling rats in these studies. And, as pointed out by Burr and Barnes ('43) one would hardly expect it to do so because of the large storage of linoleic acid and the short experimental period employed. Also supporting the view that linoleic acid is not a limiting factor in this particular case is the fact that feeding ethyl linolate to the young did not improve the rate of gain.

Quackenbush, Kummerow and Steenbock ('42) found that body fats of rats suffering from an essential fatty acid deficiency had higher iodine values than those which had received a supplement of ethyl linolate. In our study the iodine numbers of the body fats of young rats on the low-fat diet were lower than those on the corn oil diet further suggesting that a deficiency of linoleic acid was not a factor limiting growth on the low-fat diet.

Forbes and Swift ('43) have shown that the addition of fat to a diet of protein and carbohydrates decreases the heat increment and thus increases the net energy value of the diet. Such an effect could explain the results observed on the corn oil and the fat-free diet but would offer no explanation of the poor response on hydrogenated coconut oil. It is evident that the kind of fat is an important factor.

#### SUMMARY

Studies are reported in which the growth of standardized litters to 17 days of age is used as a measure of lactation performance. In these studies rats suckled by mothers fed a diet containing corn oil made more rapid growth than rats whose mothers were fed a fat-free diet. Carcass analyses showed that the extra gain of the young consisted largely of fat.

A diet containing hydrogenated coconut oil gave no better growth than the fat-free diet. No improvement in growth of the young was produced by feeding ethyl linolate to the mothers or directly to the young. Similarly, feeding the mothers 125 mg. of ethyl linolate each day did not improve the lactation response on the fat-free diet.

#### ACKNOWLEDGMENT

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# THE VITAMIN C LEVEL OF THE BLOOD PLASMA IN GUINEA PIGS

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## ONE FIGURE

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In the literature on vitamin C determinations in humans there are comments on the inadequacy of making a single, random determination of ascorbic acid in the blood and on the importance of accurately controlling the time elapsing between successive tests for the vitamin in consecutively drawn blood specimens. With the exception of the work of Rohmer et al. ('35) and Zilva ('37), none of the studies on guinea pigs considers these points. Reported normal levels of blood vitamin C values range from approximately 0.0 mg. % to 1.08 mg. %.

It is the purpose of this paper to show that the vitamin C determinations on the blood plasma of guinea pigs are of value only when the time elapsing between the intake of the vitamin and the withdrawal of the blood sample is considered; that the initial value is not a reliable index of the state of vitamin C nutrition of the animal; and that, unless the sampling error is known, a correct interpretation of the experimental data cannot be made.

## EXPERIMENTAL

Guinea pigs of both sexes, weighing approximately 250 to 450 gm., were fed for several days before being used, a diet consisting of ad libitum quantities of hay; rolled oats each 100 gm. of which was mixed with 0.25 gm. of sodium chloride, 0.5 gm. of dried brewer's yeast<sup>1</sup> and 1 ml. of U.S.P. XI. cod liver oil; greens, except on Sunday, consisting chiefly of cabbage, celery and carrots; and fresh water.

Ascorbic acid injections were made intraperitoneally in amounts regardless of body weight, since the animals were all within the same weight range.

Blood samples were obtained by lightly anesthetizing the animals with ether and withdrawing 0.5 to 1.0 ml. of blood by cardiac puncture. The survivors were used again after resting for a minimum of 3 days.

<sup>1</sup> Fleischmann.

The determinations of reduced vitamin C were made by the micro method of Farmer and Abt ('34-'35), ('36). A calibrated 0.2 ml. burette graduated in thousandths of a ml. and fitted with a stopcock and a fine capillary was used. Titrations were made with the burette held in a vertical position. Transfers of liquid of 0.2 ml. or less were made with a 0.2 ml. pipette graduated in thousandths of a ml. Determinations were made in triplicate on each sample of plasma. The accuracy of this method was  $5.4 (\pm 3.9)\%$ .

In experiment I, plasma vitamin C determinations were made without exact time controls and at different times of day (table 1).

TABLE 1

*Random determinations of vitamin C in the blood plasma of normal guinea pigs.*

NUMBER OF ANIMALS IN EACH GROUP	TIME OF DETER- MINATION AFTER FEEDING GREENS (AD LIBITUM)	PLASMA VITAMIN C MEAN $\pm$ STANDARD DEVIATION
	<i>Hours</i>	<i>Mg. %</i>
Group I. (6)	2-3	$0.53 \pm 0.26$
Group II. (30)	3-6	$0.38 \pm 0.15$
Group III. (12)	4-6	$0.72 \pm 0.51$
Group IV. (6)	15-17	$0.16 \pm 0.06$
Group V. (20)	19-20	$0.14 \pm 0.08$
Group VI. (32)	42-44	$0.16 \pm 0.11$
Total (106)	(No greens on Sunday)	
Weighted mean for all animals .....		0.30 mg. %
Standard deviation .....		$\pm 0.20$ mg. %
Standard error .....		$\pm 0.02$ mg. %

Experiments II, III, and IV, described below, indicate the rapidity with which the reduced form of the vitamin disappears from the blood.

Experiment II was divided into four parts as follows: (a) Each of nine guinea pigs on the regular diet plus greens ad libitum was injected intraperitoneally with 2.5 mg. of ascorbic acid at 0, 24, 72, and 96 hours. Twenty-four hours after the last injection of ascorbic acid, blood samples were obtained for analysis. On the day of vitamin C determination, no greens were fed. The mean value of reduced vitamin C in the plasma was  $0.07$  mg. % with a standard deviation of  $\pm 0.15$ .

(b) Each of fourteen animals was injected with 5.0 mg. of ascorbic acid at 0, 24, and 48 hours. Blood samples were taken 17 hours after the last injection. No greens were fed on the day of vitamin C determination. The plasma values were  $0.06 \pm 0.06$  mg. %.

(c) Each of six animals was administered 10.0 mg. of ascorbic acid, at which time all greens were removed. Four hours after the injection, the vitamin C content of the plasma was  $0.51 \pm 0.46$  mg. %.

(d) Six animals were fed greens at 9:45 A. M.; no injections were given these animals. Two hours later, a blood sample was drawn, and all greens were removed from the cages. The value for this sample was  $0.32 \pm 0.12$  mg. %. Five hours after the withdrawal of the greens, the mean value was  $0.08 \pm 0.04$  mg. %.

In experiment III, each of nineteen guinea pigs was injected with 10.0 mg. of ascorbic acid. At the end of 1, 3, and 7 hours after the injection, each of the animals was bled, and the reduced vitamin C content of the plasma determined. A similar experiment was conducted with eighteen animals for the time intervals of  $\frac{1}{2}$ ,  $1\frac{1}{2}$ , and 4 hours. The results appear in figure 1.

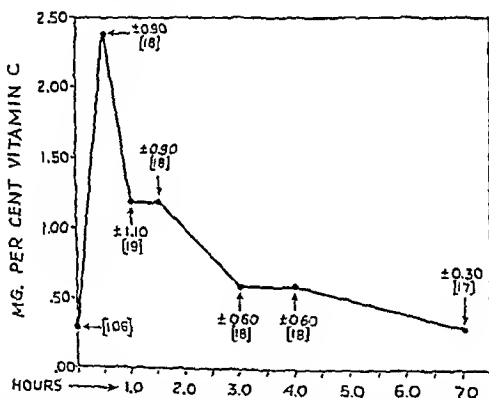


Fig. 1 Rate of disappearance of blood plasma vitamin C in guinea pigs following an intraperitoneal injection of 10.0 mg. of ascorbic acid. Numbers in parentheses represent the number of animals on each determination. Numbers indicated by arrows are the standard deviations for the respective plotted values on the graph.

In experiment IV, the effect of 20 hours of starvation was determined. Twelve guinea pigs were used. The initial plasma vitamin C level was  $0.72 \pm 0.51$  mg. %. After starvation, the value was  $0.12 \pm 0.07$  mg. %. On May 29th, the experiment was repeated with twenty guinea pigs. The initial value was  $0.14 \pm 0.08$  mg. %. After the 20 hours of starvation, the level had dropped to  $0.01 \pm 0.03$  mg. %. For all thirty-two animals before starvation, the weighted mean value was  $0.36 \pm 0.24$  mg. %; after 20 hours of starvation,  $0.05 \pm 0.05$  mg. %. This is a reduction of over 86%.



## DISCUSSION

It is apparent that the reduced vitamin C content of the plasma of guinea pigs depends on the time elapsing between the consumption of vitamin C and the withdrawal of the blood sample for the vitamin determination. In figure 1, for example, there is a significant difference of means between the values for  $\frac{1}{2}$  hour and 1 hour, between the values for 1 hour and 3 hours, and between those for 3 hours and 7 hours when "t" is greater than 2.00 (Fisher, '34).

From tables 1 and 2, it is obvious that the individual variation in the vitamin C blood plasma levels of guinea pigs is large.

TABLE 2  
*Significant difference of means for values given in table 1.<sup>1</sup>*

GROUP	I 0.53 MG.	II 0.38 MG.	III 0.72 MG.	IV 0.16 MG.	V 0.14 MG.	VI 0.16 MG.
	%	%	%	%	%	%
I 0.53 mg. %	...	1.15	1.06	<i>3.48</i>	<i>3.60</i>	<i>3.56</i>
II 0.38 mg. %	1.15	...	2.00	<i>2.34</i>	<i>2.72</i>	<i>2.48</i>
III 0.72 mg. %	1.06	2.00	...	<i>3.78</i>	<i>3.94</i>	<i>3.80</i>
IV 0.16 mg. %	<i>3.48</i>	<i>2.34</i>	<i>3.78</i>	...	0.63	0.00
V 0.14 mg. %	<i>3.60</i>	<i>2.72</i>	<i>3.94</i>	0.63	...	0.76
VI 0.16 mg. %	<i>3.56</i>	<i>2.48</i>	<i>3.80</i>	0.00	0.76	...

<sup>1</sup> Any figure in italics is considered to indicate a significant difference, since this figure gives a probability of not less than 1 in 20 that the difference would arise by chance.

## CONCLUSION

It is concluded, therefore, that a single, random determination of the reduced ascorbic acid content of the plasma of guinea pigs is not indicative of the state of vitamin C nutrition of the animal. In the determination of the state of vitamin C saturation or depletion of guinea pigs, the individual variation and the time elapsing between the last intake of the vitamin and the withdrawal of the blood sample from the animal should be considered.

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# FURTHER STUDIES ON THE VITAMIN C METABOLISM OF PRESCHOOL CHILDREN

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The data presented here are part of a larger study on the influence of changes in the ascorbic acid and citrate content of the diet on the ascorbic acid, citric acid, calcium, phosphorus, and nitrogen metabolism of preschool children. Data on the minimum amount of ascorbic acid required to maintain tissue saturation in four preschool children have been published previously (Hathaway and Meyer, '41). The present report is an extension of this earlier work on vitamin C metabolism using eight more children, and also furnishes data on the effect of supplements of ascorbic acid, potassium citrate, and orange juice on the excretion and apparent utilization of ascorbic acid.<sup>2</sup> The effect of these supplements on calcium, phosphorus, nitrogen and citric acid metabolism will be reported separately.

## EXPERIMENTAL

### *Plan of the experiment*

Two groups of four children each lived at the college laboratory-apartment for 5 months. During a preliminary period of about a week they became adjusted to their new environment, and the amount of food each child could easily consume was determined. Standardized amounts of the basal foods were consumed not only for each 7-day period, but also for the entire 20 weeks of the experiment. Supplements of crystalline ascorbic acid, potassium citrate, and orange juice were given as indicated in table 1. The amount of potassium citrate used, 3.38 gm., corresponded to the amount of citric acid and its salts calculated to be present in 200 ml. of orange juice, i.e., the amount of orange juice which is needed to supply 100 mg. of ascorbic acid.

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<sup>2</sup> A detailed report of this study is given in the dissertation submitted by Frieda L. Meyer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate School of Cornell University, October, 1943.

TABLE 1

*Average daily ascorbic acid intakes and "utilization" values by periods for eight preschool children.*

1940-41							1941-42						
PERIOD	INTAKE		"UTILIZATION"				PERIOD	INTAKE		"UTILIZATION"			
	Diet	Supplement	A	B	C	D		Diet	Supplement	E	F	G	H
	mg.	mg.	mg.	mg.	mg.	mg.		mg.	mg.	mg.	mg.	mg.	mg.
1	23	100	38	34	33	39	1	14	100	35	34	24	26
2	24	100	29	32	30	31	2	15	100	38	36	32	29
3	25	100	30	29	30	30	.	..	...	..	..	..	..
4	23	...	14	14	13	15	3	25 <sup>1</sup>	...	18	17	17	17
5	24	...	18	18	19	19	4	24 <sup>1</sup>	...	19	18	19	18
6	24	...	18	17	18	19	.	..	...	..	..	..	..
7	22	100	38	37	34	38	5	25 <sup>1</sup>	100	34	31	28	25
8	24	100	41	38	46	40	6	25 <sup>1</sup>	100	32	29	28	22
9	24	100	47	36	34	37	.	..	...	..	..	..	..
10	24	...	15	16	16	18	7	25 <sup>1</sup>	...	17	17	18	16
11	22	...	16	17	17	18	8	25 <sup>1</sup>	...	19	19	19	18
12	24	...	19	20	20	20	9	25 <sup>1</sup>	...	20	20	20	18
13	24	100	39	36	40	44	10	26 <sup>1</sup>	100	37	37	36	32
14	21	100	37	35	38	40	11	26 <sup>1</sup>	100	35	33	34	26
..	..	...	..	..	..	..	12	28 <sup>1</sup>	100	34	29	32	25
15 <sup>2</sup>	22	...	16	15	16	15	13 <sup>2</sup>	28 <sup>1</sup>	...	21	23	22	20
16 <sup>2</sup>	24	...	19	18	19	19	14 <sup>2</sup>	27 <sup>1</sup>	...	22	23	23	21
17 <sup>2</sup>	22	...	18	17	18	19	15 <sup>2</sup>	26 <sup>1</sup>	...	21	22	22	20
18 <sup>2</sup>	23	100	41	34	41	37	16 <sup>2</sup>	27 <sup>1</sup>	100	49	44	38	34
19 <sup>2</sup>	23	100	35	33	37	39	17 <sup>2</sup>	27 <sup>1</sup>	100	38	35	31	28
20 <sup>2</sup>	24	100	41	39	39	40	18 <sup>2</sup>	26 <sup>1</sup>	100	41	39	36	33
..	..	...	..	..	..	..	19 <sup>2</sup>	16	110	43	41	36	34
..	..	...	..	..	..	..	20 <sup>2</sup>	17	110	48	41	38	33

<sup>1</sup> Includes 10 mg. crystalline ascorbic acid given daily to compensate for lower ascorbic acid value of basal foods.

<sup>2</sup> 3.38 gm. of potassium citrate given daily as the estimated citrate equivalent of 200 ml. of orange juice.

<sup>3</sup> All ascorbic acid and citrate supplements given in the form of orange juice.

### *Subjects*

Eight healthy preschool children were the subjects for this study. Subjects A through D served in 1940-41, which will hereafter be referred to as the first year, and E through H served in the second year, 1941-42. Subjects A, C, F, G, and H were girls, aged 55, 49, 49, 40, and 38 months, respectively, at the beginning of the study. Their respective weights at that time were 50, 38, 40, 32, and 29 pounds. Subjects B, D, and E were boys, aged 51, 44, and 55 months, weighing 36, 35,

and 34 pounds, respectively. Physical examinations and mental tests at the beginning and end of the study showed that all eight of the children were within the normal range as judged by present standards of measurement.

### *Diets*

The basal foods were similar to those described by Hathaway and Meyer ('41), but minor alterations were made such as changes in the cereals and desserts.<sup>2</sup> In order to test the effect of changes in the ascorbic acid and citrate content of the diet at different levels of calcium intake, the milk consumption was reduced from 800 ml. per day during the first year to 500 ml. the second year. To maintain relatively constant values of the other essential constituents of the diet, the amounts of meat and eggs were increased, and peanut butter was added. The basal foods were analyzed for ascorbic acid and the values were comparable for the 2 years except for the canned potatoes. The same brand contained 18 mg. per 100 gm. the first year, and only 4 mg. the second year. To compensate for this, and also for the lowered ascorbic acid in the milk due to lowered intake,<sup>3</sup> 10 mg. of crystalline ascorbic acid were added to the basal diet during the second year.

The two younger children, G and H, were given somewhat less of the basal diet than the others, but this had little effect on their ascorbic acid intake. Milk and all supplements were given at the same level to all four children in each group.

### *Ascorbic acid determinations*

Methods of urine collection and preservation and of ascorbic acid determination were essentially the same as those described previously (Hathaway and Meyer, '41). The formula suggested by Kuether and Roe ('41) was used for the calculations during the second year, permitting simplification of the determinations of the "Gb" values.

## RESULTS AND DISCUSSION

### *Reserves of ascorbic acid*

The response to the 100 mg. supplement of ascorbic acid following periods on the basal diet was used as the means of estimating the extent of saturation of the tissues with this vitamin (see table 2). Bau-

<sup>2</sup> See footnote, page 93.

<sup>3</sup> Specially prepared by Prof. E. S. Guthrie to preserve the ascorbic acid content. See Sharp, Hand and Guthrie ('39).

mann and Rappolt ('37) considered the excretion of at least 80% of a 100-mg. test dose within 24 hours as indicative of tissue saturation. In this study 80% of the 100-mg. test dose was equivalent to 64% of the total ascorbic acid intake. None of the subjects of this study excreted more than 37% of the intake on the first day. Subjects B, E, G, and H excreted 60 to 73% in at least one test by the second day, but only H excreted over 64% in 48 hours in both tests.

TABLE 2

*Percentage of ascorbic acid excreted by the eight preschool children on the 100-mg. supplement.*

1940-41						1941-42					
SUBJECT		A	B	C	D	SUBJECT		E	F	G	H
		%	%	%	%			%	%	%	%
On days for adjustment <sup>1</sup>						On days for adjustment <sup>1</sup>					
Day	Period					Day	Period				
1st	7th	14	30	20	24	1st	5th	27	34	31	37
	13th	17	22	9	15		10th	10	15	19	28
2nd	7th	46	60	52	55	2nd	5th	55	41	55	72
	13th	52	63	38	49		10th	64	58	69	73
3rd	7th	64	66	67	66	3rd	5th	72	70	75	76
	13th	58	67	62	67		10th	63	67	71	70
4th	7th	73	72	76	76	4th	5th	84	73	74	77
	13th	67	70	66	69		10th	84	71	69	74
On days after adjustment <sup>2</sup>						On days after adjustment <sup>2</sup>					
Mean		70	72	71	69	Mean		73	75	75	79
Range		(53-81)	(64-79)	(50-84)	(55-82)	Range		(62-84)	(68-86)	(67-83)	(70-87)

<sup>1</sup> Days immediately following periods on the basal diet only.

<sup>2</sup> All but the first two days on the 100 mg. ascorbic acid supplement.

Since 125 mg. of ascorbic acid greatly exceeds the allowance recommended for children of this age range, the excretion value exhibited after adjustment to this intake may be used as a criterion for saturation. The excretion values for these eight children after such adjustment ranged from 50 to 87%. This adjustment required at least 2 days for any child if his lowest excretion value after adjustment is used as the criterion, and 3 to 4 days if his mean excretion value is used.

Further evidence that the tissues were probably not saturated when the subjects were on the basal diet alone is given by comparison of the "utilization"<sup>4</sup> values (table 1) with results from the previous

<sup>4</sup> The term "utilization" is used arbitrarily to refer to the difference between intake and excretion.

study (Hathaway and Meyer, '41; table 4). In the earlier study it was shown that the lowest intake which would maintain tissue saturation was 31 mg. and on this level of intake the "utilization" values averaged 22 mg. The intake on the basal diet in this study was only 23 to 25 mg. and the "utilization" values averaged 18 mg. Since the highest average per cent "utilization" found in any of the twelve children was 80%, one should not expect 22 mg. to be utilized on a 23- to 25-mg. intake. Consequently it may be concluded, using any of these criteria, that the tissues were not completely saturated. The 23- to 25-mg. intake, however, was not far below the amount needed for tissue saturation.

TABLE 3

*Means of ascorbic acid "utilization" values and corresponding urinary pH values.*

1940-41								
SUBJECT	"UTILIZATION" ON BASAL DIET	pH	"UTILIZATION" ON BASAL DIET PLUS POTASSIUM CITRATE	pH	"UTILIZATION" ON 100-MG. SUPPLEMENT	pH	"UTILIZATION" 100-MG. SUPPLEMENT PLUS POTASSIUM CITRATE	pH
	mg.		mg.		mg.		mg.	
A	17 ± 2.0	5.7	18 ± 1.1	6.8	37 ± 8.0	5.8	39 ± 7.3	6.9
B	17 ± 1.9	6.0	17 ± 1.5	6.8	35 ± 5.0	5.9	35 ± 6.0	6.8
C	18 ± 2.2	5.7	18 ± 1.7	6.7	35 ± 7.6	5.7	39 ± 8.8	6.7
D	18 ± 1.6	5.6	18 ± 2.0	6.7	37 ± 7.6	5.6	39 ± 5.3	6.7
1941-42								
E	19 ± 1.6	5.8	21 ± 1.2	6.8	34 ± 6.4	5.7	42 ± 6.6	6.9
F	18 ± 1.7	5.9	22 ± 1.3	6.9	32 ± 6.7	5.9	39 ± 7.2	6.8
G	18 ± 1.5	5.7	22 ± 1.3	6.9	32 ± 6.1	5.6	35 ± 6.6	6.9
H	17 ± 1.5	5.6	20 ± 1.1	6.9	26 ± 6.5	5.5	31 ± 4.7	6.9

*The effect of potassium citrate on the "utilization"  
of ascorbic acid*

The mean "utilization" values with the standard deviations are given in table 3. On the basal diet alone the eight children had similar "utilization" values. When potassium citrate was added, the individual responses of the children in the first group were significantly increased in only two cases, subject A on the basal diet, and subject C on the 100-mg. ascorbic acid supplement. With the second group of children, however, the increased "utilization" of ascorbic acid with the addition of potassium citrate was highly significant in every case except G when she was on the 100-mg. ascorbic acid supplement. When the data for the first group were treated by analysis of variance there

was no significant difference in "utilization" between the periods with and without the potassium citrate supplement, but with the data from the second group the odds were 99:1 against the chance occurrence of so great an increase in "utilization."

There is no apparent explanation for the difference in response between the two groups of children. Changes in urinary pH cannot account for the difference, since the increase in pH due to the administration of potassium citrate was of the same order in all the subjects (see table 3).

It is possible that changes in the basal foods might account for the difference in response to citrates. Although the percentage of protein in the diet was the same for both groups, the sources of the protein were different. The decrease in milk and the increase in meat and eggs the second year resulted in the following changes (calculated values) in essential food factors of the basal diet: the calcium was reduced from 1.1 to 0.8 gm., the riboflavin from 1.8 to 1.3 mg. and the acid: base ratio from 1:1.8 to 1:1.2.

Purinton and Schuck ('43) have suggested that there may be a relationship between ascorbic acid and citric acid metabolism. The urinary excretion of citric acid in children E, F, G, and H was determined by Metcalf ('43). Changes in citric acid excretion were associated with changes in urinary pH rather than with changes in ascorbic acid excretion.

From the data on these two groups of children there is some indication that addition of potassium citrate to the diet causes an increase in "utilization" of ascorbic acid. The individual differences in response in the first group of children were not mathematically significant in most cases, but when potassium citrate was added to the diet the "utilization" of ascorbic acid was equal to or greater than the "utilization" without citrate in all cases. There were apparently other factors than citrate administration or changes in urinary pH which affected the "utilization" of ascorbic acid. Changes in the high protein foods of the basal diet may have been a factor, but differences in metabolic reactions of the individual children seem more important.

*The effect of orange juice on the "utilization"  
of ascorbic acid*

Average ascorbic acid "utilization" values for the two periods during the second year when orange juice was used as a supplement to the basal diet are included in table 1. Higher "utilization" was found for all four children. When the data for the "utilization" values on the

crystalline ascorbic acid supplement and the orange juice supplement were treated by analysis of variance the odds were greater than 99:1 against the chance occurrence of so great an increase in "utilization". When the corresponding data on the ascorbic acid plus potassium citrate supplement were compared with those on the orange juice supplement, the odds were still greater than 19:1 that the ingestion of orange juice was associated with greater "utilization" of ascorbic acid.

The average pH values of the urine during the periods on the orange juice supplement for the four children ranged from 6.0 to 6.2. These values were higher than the corresponding values during the periods on crystalline ascorbic acid (5.5 to 5.9), but were lower than for the periods on potassium citrate (6.8 to 6.9). Thus changes in "utilization" values did not vary directly with the pH changes.

Studies to compare the "utilization" of ascorbic acid in foods with the "utilization" of equivalent amounts of crystalline ascorbic acid are always complicated by the addition of other food factors than those to be tested. Jacobsen ('35), and Hawley et al. ('37) found better storage of ascorbic acid in the tissues when it was administered as a food than when it was given in the crystalline form. Todhunter, Robbins, Ivy, and Brewer ('40) and Todhunter and Fatzer ('40), on the other hand, concluded from both animal and human studies that the response to both forms of ascorbic acid was the same.

Although there was a significant increase in the "utilization" of ascorbic acid in these four children when orange juice was the source of the vitamin, it should not be concluded that the ascorbic acid per se was better utilized until this observation is confirmed by further studies. In these same children potassium citrate, also a constituent of orange juice, improved the "utilization". It may have been an individual response to the potassium ion, to the citrate ion, to changes in acid-base balance, or to some unrecognized food factor which was responsible for the improved "utilization" on the orange juice supplement rather than a difference in the natural and crystalline ascorbic acid. Since the "utilization" values on the orange juice supplement were significantly higher than with potassium citrate added to crystalline ascorbic acid, however, it seems probable that there was some other factor than potassium citrate which was at least partially responsible for the increased "utilization".

#### SUMMARY

The ascorbic acid metabolism has been studied in eight preschool children. The basal diet containing 23 to 25 mg. ascorbic acid was



supplemented with 100 mg. ascorbic acid, 3.38 gm. potassium citrate, or equivalent amounts of orange juice as indicated. The results were as follows:

1. A daily intake of 23 to 25 mg. of ascorbic acid was not sufficient to maintain tissue saturation in the eight children.

2. On the addition of 3.38 gm. potassium citrate to the diet "utilization" of ascorbic acid was increased in five of the eight children at both levels of ascorbic acid intake (25 and 125 mg.), and in two others at the higher level of ascorbic acid intake.

3. On the substitution of orange juice for crystalline ascorbic acid and potassium citrate, the "utilization" of ascorbic acid was significantly increased in the four children studied.

4. The "utilization" of ascorbic acid was not directly related to the urinary pH.

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# THE NUTRITIVE VALUE OF CANNED FOODS

## I. INTRODUCTION AND SAMPLING PROCEDURE

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The studies of Kohman and Eddy ('37), sponsored by the National Canners Association during the period 1924-1937, produced information on the nutritive value of canned foods as determined mainly by animal assay methods. Subsequently, further fractionation of the vitamin B complex into well established entities, together with the development of improved methods for their determination and more suitable methods for the determination of other vitamins, emphasized the need for further work. This need was further highlighted by the National Nutrition Conference for Defense in May, 1941. At that time available critical reviews (Daniel and Munsell, '37) contained only limited information on the nutritive values of canned foods. It was obvious that the focus of attention on the nutritional problems of our Armed Forces and civilian population would create new demands for accurate and extensive information on all types of food products, canned foods included.

In recognition of this important need, a nutrition program sponsored jointly by the National Canners Association and the Can Manufacturers Institute was organized in early 1942. This program was administered by an Executive Committee, later assisted by an Industry Advisory Committee. A program of investigation requiring several years for completion was laid out. In broad aspect, this program included: complete information on the nutritive value of commercially canned foods; the effects of preparation methods used in the home, restaurant, and Army kitchens; and the effects of time and temperature of storage on certain vitamins in canned foods. Further, projects were outlined in which the influence of specific canning operations on vitamins in raw foods were to be studied to determine where improvements in existing canning practices might possibly be made.

The 1942 program included the assay of 823 samples of commercially canned foods representing thirty-two products for the factors, ascorbic acid, carotene, riboflavin, niacin, thiamine, and pantothenic acid. Grants

were allocated to various universities where the vitamin assays were carried out under the guidance of authorities in nutritional studies. By correspondence and conference, the methods of assay for the factors were discussed and in all instances the chemical or microbiological methods which were considered to be most applicable were selected. The necessity for correlation studies in which the chemical and microbiological methods could be correlated with officially accepted methods of animal assay was obvious. Arrangements were made to include such work in the first year's program. Studies on the effects of home and large scale kitchen practices (Army and Navy) on the vitamin content of canned foods were initiated. Preliminary investigation was also outlined on the effect of storage on the vitamin content of canned foods. Proximate and mineral analyses (calcium, phosphorus, and iron) of a representative number of the samples of each product were undertaken. Specifically, the 1942 program required the coordinated cooperation of the Universities of Wisconsin, Texas, Chicago, Arizona, Maryland, and Pennsylvania State College. Portions of the program carried out by these institutions under grants-in-aid will be disclosed in subsequent publications.

In consideration of the products which should be assayed during the first year of this program, it was decided that, as a general principle, only non-formulated canned products of nutritional significance, which were produced and consumed in relatively large quantities, should be included. Thirty-two products were selected for the first year's study, including one formulated product, baked beans, because of the Army's interest in it. Samples of these products were taken mainly from the 1942 packs, and, in a few instances, from the spring packs of 1943. The sampling program was organized so that samples of the products in retail size cans (mainly no. 2 and no. 2½ sizes) and of some products in no. 10 cans would be taken from all of the principal canning areas for each specific product. Two or three sampling periods, mid-early and mid-late, or early, middle, and late season samplings, respectively, were planned when seasonal variations in the products might be expected.

The samples were, in most instances, taken from canneries by representatives of the industry designated to collect the samples. Every attempt was made to obtain "run of the mill" samples, in order to provide as good indications as possible of the nutritive value of the major products canned in the United States. It was felt that maximum and minimum vitamin values obtained on a large number of samples of the same product would serve to fix more closely the ranges of vitamin

content for the specific products as well as increase the reliability of the figures for average vitamin contents. In table 1 are shown both the products selected and the scope of sampling.

One sampling at a plant for one period of the season consisted of thirty-six retail size cans or six to twelve no. 10 cans. Where samplings of 46-ounce cans were taken, eighteen cans comprised a sampling. A sampling represented a day's pack of a product in one can size, and, in most instances, consisted of an equal number of cans from the early, middle, and late portion of a day's pack. A complete history of the samples was obtained at the time of sampling, which included all of the information possible about the canned product, raw product used, and the canning procedure employed. It was believed that an analysis of the vitamin and historical data on the samples would reveal some trends in the results which might be of value in future studies of vitamin retention.

The samples were sent to a central sample receiving and distribution point, where they were coded for proper identification, inspected, and divided for shipment to the various laboratories. Two retail size cans or one no. 10 can from each sampling were checked for vacuum and headspace. Net weight, drained weight, pH and general quality characteristics of the products were determined. Six retail size cans or one no. 10 can from each sampling were sent to each university conducting the vitamin assays. Some of the samples of certain products in retail and no. 10 can sizes were used for study of the distribution of water soluble nutrients between the solid and liquid portions of the can contents, and for studies on the effect of preparation for serving on the vitamin content. The remaining samples were held in storage as duplicate samples, some being used for the proximate and mineral analyses.

The six retail size cans of each sampling received by each collaborator were combined into one composite sample and representative portions taken for the assay of two vitamin factors. Thus each analysis of a sampling of the retail size cans included a sufficient number of cans and quantity of product to give a good indication of the vitamin values on a day's pack at a given plant. When more than one sampling of no. 10 cans of a product were taken at one plant during the season, two or three no. 10 cans, one from each sampling, were combined into one composite sample.

The vitamin values obtained by the collaborators on the canned samples are reported in the accompanying papers. The reports on the other work undertaken in this program will appear in subsequent publications.

TABLE 1  
Products collected from 1942 packs for determination of nutritive value and composition.

PRODUCT	NO. OF PLANTS SAMPLED	EASTERN DISTRICT				MIDWESTERN DISTRICT				WESTERN DISTRICT			
		Retail size cans		No. 10 cans		Retail size cans		No. 10 cans		Retail size cans		No. 10 cans	
		No. of plants	No. of samples	No. of plants	No. of samples	No. of plants	No. of samples	No. of plants	No. of samples	No. of plants	No. of samples	No. of plants	No. of samples
Apricots, unpeeled halves	8	..	..	..	2	..	6	4	4	13	21	5	5
Asparagus, all green	15	3	7	2	2	..	..	..	..	5	7	4	4
Asparagus, culturally bleached	4	..	..	..	..	..	..	..	..	4	8	4	4
Beans, baked New England style	3	..	..	2	2	..	..	..	..	..	..	1	1
Beans, baked with tomato sauce	2	..	..	1	1	..	..	1	1	..	..	..	..
Beans, green cut	23	3	9	3	3	10	29	10	10	6	16	6	6
Beans, lima, green	14	11	13	7	7	3	5	2	2	..	..	..	..
Beets	9	3	6	3	3	4	8	4	4	2	4	2	2
Carrots	7	2	4	2	2	3	6	3	3	2	2	2	2
Corn, white, whole kernel	15	4	12	2	2	10	28	4	4	..	..	..	..
Corn, yellow, whole kernel	24	3	9	3	3	12	31	9	9	2	6	2	2
Grapefruit juice	20	8	18	..	..	9	18	..	..	3	7	..	..
Grapefruit segments	10	8	19	..	..	2	5	..	..	..	..	..	..
Mackerel	8	2	3	..	..	..	..	..	..	6	6	..	..
Orange juice	8	3	4	..	..	..	..	..	..	5	11	..	..
Peaches, halves (clingstone)	9	..	..	..	..	..	..	..	..	9	17	..	..
Peaches, halves (freestone)	11	1	2	..	..	1	2	..	..	9	9	..	..
Pears, halves	15	1	2	..	..	2	4	..	..	12	24	..	..
Pears, sweet (wrinkled varieties)	26	6	18	4	4	11	31	10	10	8	24	5	5
Pens, Alaska	2	..	..	..	..	2	6	1	1	..	..	..	..
Pineapple juice	8	..	..	..	..	..	..	..	..	8	13	..	..
Pineapple, sliced	8	..	..	..	..	..	..	..	..	8	17	..	..
Prunes, Italian	5	..	..	..	..	..	..	..	..	5	10	..	..
Salmon	5	..	..	..	..	..	..	..	..	5	5	..	..
Sardines, in oil	5	5	5	..	..	..	..	..	..	..	..	..	..
Sardines, in tomato sauce	10	..	..	..	..	..	..	..	..	10	10	..	..
Shrimp, dry pack	3	3	3	..	..	..	..	..	..	..	..	..	..
Shrimp, wet pack	5	5	5	..	..	..	..	..	..	..	..	..	..
Spinach	19	3	3	2	2	5	5	2	2	11	12	7	7
Tomatoes	26	6	17	..	..	7	18	1	1	10	23	2	4
Tomato juice	30	12	35	..	..	8	23	..	..	10	21	..	..
Tuna	6	..	..	..	..	..	..	..	..	6	6	..	..
Total	303	92	194	31	31	95	223	51	51	169	284	36	38

## ACKNOWLEDGMENT

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cans in the same group were then obtained by blending the total can contents in the Waring Blendor from 15 seconds to 2 minutes, the time depending upon the character of the material. Suitable aliquots of the comminuted material were weighed immediately and prepared for analysis.

### *Analytical methods*

The vitamin assay methods selected for use in this study are among those recommended by the Committee on Vitamin Assay Methods of the National Cooperative Experiment Station Project on Conservation of the Nutritive Values of Foods.

*Vitamin C.* The ascorbic acid content of all the canned foods was determined following the chemical method of Bessey ('38) and modified by Morrell ('41) briefly described as follows: Twenty-five gram samples, of the comminuted solids and liquids or 25 ml. of the canned juices (tomato, grapefruit, orange, and pineapple) were placed in 100 ml. volumetric flasks and brought up to volume with 3% metaphosphoric acid solution. In general, the contents of the flasks were thoroughly shaken and then filtered through dry, fluted Whatman no. 12 filter paper. Only in the case of corn which, because of the toughness of its hull, was found more difficult to homogenize, did better extraction of the ascorbic acid result from the use of the Waring Blendor. Separation of the solid material of corn was also better accomplished by centrifuging.

Aliquots of the almost clear filtrate containing from 100 to 200  $\mu$ g. of ascorbic acid were transferred to 50-ml. volumetric flasks and their pH adjusted to approximately 3.6 with a sodium citrate buffer. This mixture was then brought up to volume and to a final pH of  $3.6 \pm 0.1$  with a sodium hydroxide, citric acid, and metaphosphoric acid buffer solution. Suitable size of the aliquots selected for the different canned foods was found to range from 1 to 2 ml. for the citrus fruits; 3 to 5 ml. for asparagus, spinach, and tomatoes; 5 ml. for peas, pineapple juice, and lima beans; and 10 to 15 ml. for peaches, pears, and most of the other canned foods of low vitamin C value. The bleaching effect of 5 ml. of this solution on 5 ml. of a solution of the dye 2, 6-dichlorobenzenoneindophenol (20 mg. per liter of water) was then measured, following in detail the Morell ('41) method using the Evelyn Photoelectric Colorimeter with the green filter no. 520. Correction was made for interference caused by the turbidity and the color of the unknown test solutions in the usual manner. In the case of beets and prunes, the size of the aliquot used was necessarily limited to 5 ml. in spite of their low ascorbic acid content because of the excessive in-

interference caused by the deepness of their color. Only in the case of asparagus was there observed a rapid rate of fading and therefore a high rate of drift in the galvanometer readings after the addition of the dye solution. When correction was made for this by subtraction of the difference between the 15-second and the 30-second readings as proposed by Bessey ('38), duplicate samples checked within limits of the accuracy of the method.

The colorimeter readings were interpreted from calibration curves, made from standard solutions of pure ascorbic acid.

*Vitamin A.* The vitamin A value of all the canned fruits and vegetables was measured by their pro-vitamin A or carotene content following the chemical assay procedure of Moore ('40) and modified by Moore and Ely ('41). Briefly described, the carotene in comminuted solid and liquid samples was extracted in a mixture of approximately 57% of 95% ethyl alcohol and 43% of a petroleum ether solution<sup>3</sup> by blending for 5 minutes in a Waring Blendor. The two liquids in the mixture were then completely separated in a separatory funnel by the addition, if necessary, of enough water to make the alcohol concentration about 80% by volume. The alcoholic phase was then drawn off and the residue washed three or more times with 30-ml. portions of Skellysolve B. The combined extract was subsequently washed six times with tap water in order to remove all of the alcohol and then made up or reduced to convenient volume as indicated by depth of the color. Pigments other than carotene were removed by passing the carotene extracts through tubes containing dicalcium phosphate as the absorbent material. The filtrate was then concentrated if necessary in a hot water bath (70° C.) under reduced pressure and made up to a suitable volume. The carotene content was then measured using the Evelyn Photoelectric Colorimeter with filter no. 440. Readings were interpreted from a previously prepared calibration curve based on standard solutions of pure beta-carotene dissolved in Skellysolve B. A standard solution of potassium dichromate was used periodically to check the calibration of the colorimeter.

In this study the size of the sample selected for analysis which was found to be most convenient for several reasons was as follows: 10 gm. of foods of high carotene content, carrots, apricots, and spinach; 30 to 35 gm. of foods very low in carotene content, such as baked beans, beets, pineapple, grapefruit, orange juice, pears, and lima beans, etc.; and 15 to 25 gm. of all the other fruits and vegetables, all being weighed

<sup>3</sup> Skellysolve B, Skelly Oil Company, Chicago, Illinois.



to 0.01 gm. When even large samples of the foods very low in carotene content were extracted it was found necessary to evaporate the extracted solutions almost to dryness. The entire extract was then passed through the dicalcium phosphate column by suction in 4 to 5 minutes, washed with Skellysolve B and brought up to 10- to 25-ml. volumes, in order to have colorimeter readings fall within the most sensitive portion of the calibration curve. However, the samples of most of the foods taken were large enough to yield extracts sufficiently rich in carotene to make it unnecessary to reduce the volume by evaporation to such a great extent. Instead, suitable aliquots of the extract rather than the entire amount could be passed through the dicalcium phosphate column. Thus, a saving not only of time but of solvent was effected. It was not necessary to reduce the volumes of the carotene extract of apricots, carrots, or spinach but instead they were increased to a volume of 200 to 250 ml. and 10-ml. aliquots taken for analysis. Volumes of the extracts of most of the other foods were reduced to 50 or 100 ml. from which 20- to 25-ml. aliquots were taken to pass through the absorbent.

In most cases Baker's Analyzed C. P. dicalcium phosphate was found to be a suitable absorbent of the pigments other than carotene and to permit a 98 to 100% recovery of pure beta-carotene. It was found necessary to control the speed of passage of the extracted material by the addition of Dyno<sup>4</sup> in some cases but not in others, and to vary the height of the column. For example, the dicalcium phosphate column had to be longer than usual for the complete removal of interfering pigments from corn and peaches and to prevent them from being washed into the filtrate. Care was taken to pack the tubes evenly to prevent channeling. The greatest difficulty of all was encountered in the case of tomatoes because of the incomplete absorption of lycopene when Baker's material was used as the absorbent. The most satisfactory absorption of lycopene was obtained when lots of dicalcium phosphate prepared in this laboratory following the method of Moore ('42) were used. The reason for this is not understood. Only certain batches of this laboratory prepared material were found to be satisfactory. It was noted that the absorbing ability or activity of the product varied with the yield. For example, small yields of a very fine material held back all of the lycopene but also did not permit the passage of the carotene. On the other hand, certain large yields of a very coarse granular material which permitted the passage of carotene also allowed most of the lycopene to pass through. It was also noted that activated di-

<sup>4</sup> Corn Products Refining Company, 17 Battery Place, New York.

calcium phosphate frequently became inactive after a period of time in storage.

The vitamin A in canned fish has been determined following in general the Carr-Price method ('26) as modified by Dann and Evelyn ('38) for use with the Evelyn Photoelectric Colorimeter. Cans of tuna fish, sardines which were packed in oil, and wet pack shrimp were drained and only the solids were used for analysis as directed. Assays of salmon, mackerel, and dry pack shrimp were made on combined can contents. Analyses were made of 20- to 25-gm. portions of the fish paste weighed into Erlenmeyer flasks. Five milliliters of a saturated solution of potassium hydroxide and 20 ml. of methyl alcohol were added for every 10 gm. of fish, and the samples were refluxed for 10 minutes on a water bath and cooled under tap water. The mixture was then transferred to a separatory funnel and the vitamin A extracted by shaking it three times with 50-ml. portions of ethyl ether. The ether fractions were combined and washed with tap water seven to eight times until the wash water was neutral to litmus paper. The extract was then dried by passing through anhydrous sodium sulfate in a sintered glass funnel and evaporated to dryness under reduced pressure. Traces of moisture were expelled by placing the flask in the oven at 100° C. for 30 seconds. The unsaponifiable residue was then transferred and brought up to 10-ml. volume with dry chloroform. In measuring the vitamin A content of the test solution, 1 ml. was placed in the Evelyn colorimeter tube using filter no. 620 and antimony trichloride as the blank, and 9 ml. of antimony trichloride reagent (25 gm. antimony trichloride in 100 ml. chloroform) quickly added. Readings were made as the galvanometer pointer rested briefly before the color began to fade and the needle began to drift. The readings were interpreted by means of a calibration curve made with standard solutions of pure crystalline vitamin A alcohol<sup>5</sup> dissolved in chloroform.

Duplicate determinations were made on all samples of canned fruits and vegetables until the reliability of the method for each food was believed to be established. Great difficulties were encountered in the analyses of fish and the lack of close agreement between duplicates makes the values reported for fish more open to question.

#### RESULTS

The vitamin assay data summarized as to range and average for each of the different types of fruits and vegetables are presented in table 1.

<sup>5</sup> Distillation Products, Inc., Rochester, New York.

TABLE 1

*Ascorbic acid and carotene content of commercially canned fruits and vegetables.*

FOODS	ASCORBIC ACID			CAROTENE		
	Number of composite samples analyzed	In 100 gm. combined solids and liquids		Number of composite samples analyzed	In 100 gm. combined solids and liquids	
		Range mg.	Average mg.		Range mg.	Average mg.
Apricots, unpeeled halves	21	1.1- 5.7	3.9	21	0.93-1.74	1.28
Asparagus, all green	30	5.4-25.3	15.2	30	0.22-0.50	0.31
Culturally bleached <sup>1</sup>	12	11.6-18.1	14.9	12	Trace-0.06	0.03
Beans, baked						
New England style	3	1.9- 4.3	2.9	3	Trace-0.05	0.02
With tomato sauce	2	1.8- 3.5	2.7	2	0.04-0.06	0.05
Beans, green cut	73	0.6- 7.3	3.2	73	0.08-0.34	0.18
Beans, lima, green	26	2.5-12.1	7.0	26	0.03-0.18	0.07
Beets	27	Trace-4.0	2.0	27	Trace-0.05	0.006
Carrots	19	1.0- 4.6	2.0	19	4.47-9.44	7.16
Corn, white, whole kernel	45	2.0- 6.5	4.4	28	Trace-0.11	0.02
Yellow, whole kernel	61	1.3- 8.0	4.2	61	0.04-0.16	0.09
Grapefruit juice	43	26.3-44.7	33.8	18	Trace-0.02	0.007
Grapefruit segments <sup>2</sup>	22	20.3-32.3	24.6	3	Trace-0.02	0.009
Orange juice	14	33.0-52.4	39.4	14	0.01-0.12	0.08
Peaches, halves, clingstone	17	2.0- 5.7	3.9	17	0.19-0.51	0.26
Freestone	13	1.4- 3.7	2.3	13	0.09-0.28	0.16
Pears, halves	30	Trace-2.5	1.5	4	Trace-0.002	
Peas, sweet,						
wrinkled varieties	91	3.1-13.8	8.8	91	0.15-0.51	0.26
Alaska	7	7.4-13.7	10.0	7	0.21-0.31	0.25
Pineapple juice	18	3.2-14.2	8.5	18	0.01-0.04	0.03
Pineapple, sliced	17	0.8- 0.7	5.1	17	0.01-0.04	0.03
Prunes, Italian <sup>3</sup>	10	Trace-3.0	1.1	10	0.44-0.89	0.63
Spinach	31	3.4-25.9	11.4	31	1.68-4.81	3.16
Tomatoes <sup>4</sup>	61	9.5-27.1	16.5	61	0.35-0.96	0.58
Tomato juice	79	2.5-25.2	12.9	79	0.16-0.96	0.51

<sup>1</sup> Culturally bleached asparagus is asparagus so cultivated that the development of chlorophyll is inhibited. The color of canned culturally bleached asparagus may be practically white, yellow-white, or the heads may be yellow-green, green, or purple.

<sup>2</sup> Two cans of "pink" grapefruit omitted because the values were not within the normal range for carotene.

<sup>3</sup> Edible portion, skins and pulp, analyzed.

<sup>4</sup> Seeds included.

Vitamin A is expressed in terms of milligrams of carotene per 100 gm. of combined solids and liquids, or 100 ml. of the canned juices.

Vitamin C is expressed in terms of milligrams of ascorbic acid in 100 gm. of combined solids and liquids, or 100 ml. of the canned juices.

Table 2 presents the results of the vitamin assay of canned fish, expressed in terms of milligrams of pure vitamin A alcohol per 100 gm.

TABLE 2  
Vitamin A content of canned fish.

FOODS	NUMBER OF COMPOSITE SAMPLES ANALYZED	VITAMIN A VALUE MO. PER 100 GM.	
		Range	Average
Mackerel <sup>1</sup>	8	0.015-0.054	0.029
Salmon, red species	3	0.078-0.102	0.087
Pink species	3	0.015-0.023	0.019
Sardines, in oil	5	0.021-0.123	0.069
In tomato sauce <sup>1</sup>	10	.. ..	....
Shrimp, dry pack	3	0.014-0.023	0.017
Wet pack	5	0.015-0.022	0.018
Tuna <sup>1</sup>	6	0.005-0.010	0.008

<sup>1</sup> Oil drained off.

<sup>2</sup> Assays of sardines in tomato sauce not included in report because of unreliability of method of analysis due to interfering pigments.

It may be seen in table 1 that on the basis of their average ascorbic acid content, different kinds of canned fruits and vegetables may be grouped as follows:

Canned orange and grapefruit juices, as expected, are the best sources of vitamin C of the canned foods studied. The average ascorbic acid content was found to be 39.4 mg. per 100 ml. for orange juice and 33.8 mg. per 100 ml. for grapefruit juice. It was found that canned grapefruit segments in sirup were lower in ascorbic acid value, showing an average of 24.6 mg. per 100 gm.

Tomatoes, all green asparagus, culturally bleached asparagus, tomato juice, and spinach were found to lie in the range of 16.5 to 11.4 mg. per 100 gm. or ml.

The average ascorbic acid content of peas (including Alaska's), pineapple juice, and lima beans fell within the range of 8.9 to 7.0 mg. per 100 gm. or 100 ml. of the canned contents. The ascorbic acid content of canned sliced pineapple in sirup was relatively lower than pineapple juice alone, for it was found to yield 5.1 mg. per 100 gm.

White corn, yellow corn, clingstone peaches, apricots, green beans, baked beans, freestone peaches, beets, carrots, pears, and prunes are the poorest sources of vitamin C, yielding in the order named on the average from 4.4 to 1.1 mg. of ascorbic acid per 100 gm. combined solids and liquids.

On the other hand, when considered from the standpoint of their vitamin A value, the same commercially canned goods fell into the following groups:

The best sources of carotene among the canned foods which were tested were carrots, spinach, and apricots. The average carotene content of the combined solid and liquid of these foods was found to be 7.16, 3.16, and 1.28 mg. per 100 gm., respectively.

Prunes (pulp and skins), tomatoes, tomato juice, all green asparagus, peas, and clingstone peaches were found to be lower in carotene and to range on the average from 0.63 to 0.26 mg. per 100 gm. or ml.<sup>1</sup>

Green beans, freestone peaches, yellow corn, orange juice, and lima beans were found to range from 0.18 to 0.07 mg. per 100 gm. Canned red salmon and sardines (table 2) are the only canned fish that showed vitamin A values approximately of this order.

Culturally bleached asparagus, pineapple juice, sliced pineapple, baked beans, and white corn showed a range in average carotene content of 0.03 to 0.02 mg. per 100 gm. or 100 ml.

Grapefruit segments, grapefruit juice, beets, and pears are very poor sources of pro-vitamin A, ranging from 0.009 mg. to a trace per 100 gm. of combined solids and liquids.

Unfortunately, it is common practice for the liquid present in certain types of canned vegetables to be discarded when served. Fortunately, however, from the standpoint of vitamin A value, carotene is not highly water soluble and therefore is retained to a large extent in the food solids. In table 3, therefore, the carotene content of the canned foods, which in table 1 have been shown to be the best sources of this vitamin, has been expressed on the solid basis. This has been

TABLE 3  
*Carotene content of solid portions of certain canned foods.<sup>1</sup>*

FOODS	NUMBER OF COMPOSITE SAMPLES ANALYZED	APPROXIMATE PERCENT OF SOLIDS IN CAN <sup>2</sup>	ESTIMATED CAROTENE CONTENT OF 100 GM. OF SOLIDS	
			Range mg.	Average mg.
Apricots, unpeeled, halves	21	60	1.56- 2.90	2.13
Asparagus, all green	30	64	0.34- 0.78	0.48
Beans, green, cut	73	62	0.13- 0.55	0.30
Beans, lima, green	26	67	0.05- 0.26	0.11
Carrots	19	68	6.57-13.88	10.54
Corn, yellow, whole kernel	61	66	0.05- 0.25	0.14
Peaches, halves, clingstone	17	66	0.29- 0.78	0.39
Peaches, halves, Freestone	13	65	0.13- 0.43	0.24
Peas, sweet, wrinkled varieties	91	65	0.24- 0.78	0.40
Prunes, Italian	10	51	0.86- 1.75	1.23
Spinach	31	69	2.44- 6.97	4.58

<sup>1</sup> Based on the assumption that all carotene is in the solid portion.

<sup>2</sup> Calculated from average weights of solids and liquids in each individual can contents.

estimated on the assumption that all of the carotene is present in the solid and none in the liquid portion. Canned peaches, apricots, and prunes, fruits which are good sources of carotene, have also been included in this table, although it is customary to serve canned fruits with their sirup.

#### DISCUSSION

The number of composited samples which were assayed for ascorbic acid and carotene ranged for fruit from the minimum of 10 samples of prunes to 65 of grapefruit segments and juice and for vegetables from the minimum of 26 samples of lima beans to 140 samples of tomatoes and tomato juice. It may be noted that the range in ascorbic acid and carotene content of many of these foods is very wide. This difference, of course, is of practical importance only when the food is a good source of the vitamin in question.

Obviously differences in the vitamin values of different samples of any of these canned foods should be expected for the many samples of each kind of food analyzed include different varieties, foods grown under different soil and climatic conditions, in different parts of the country, selected from early, middle, or late parts of the season's pack, processed by different canners, and packed in cans of different sizes with some differences in the ratio of solid to liquid can contents.

It may be noted, for example, that canned clingstone peaches were found to be somewhat better sources of both ascorbic acid and carotene than freestones. Canned red salmon proved to be substantially richer in vitamin A than pink salmon, and again, canned yellow corn was found to be far richer in carotene than white corn, although there was no significant difference in their ascorbic acid content. As expected, culturally bleached asparagus was very much lower in carotene content than the same variety of asparagus so cultivated that the development of chlorophyll was not inhibited (all green). Again, however, there was no significant difference in their ascorbic acid content. The wide range of the average carotene values of canned culturally bleached asparagus (although relatively insignificant because it is a poor source of carotene) appeared to be associated with the variation in the chlorophyll content as indicated by some cans containing green-tipped heads, as contrasted with others in which the color of the asparagus heads was practically white or yellow-white.

Samples of both grapefruit segments and pineapple slices were found lower in ascorbic acid value than the canned juices of these fruits. This is to be expected because of the dilution resulting from the addition of sirup to the fruit packs. On the other hand, the samples of canned tomato juice appeared to be somewhat lower in ascorbic

acid content than the canned tomatoes. Since no diluting medium such as sirup is added in the canning of tomatoes, these differences may perhaps be associated with differences in the canning procedure.

The wide range in both the ascorbic acid and carotene values reported in table 1 for some of the canned foods such as tomatoes and tomato juice, orange juice, spinach, carrots, etc., may perhaps be associated with differences in variety and section of the country in which they were grown and processed.

#### SUMMARY

Certain commercially canned foods have been assayed for their ascorbic acid and carotene or vitamin A values following recommended chemical methods.

The ascorbic acid and carotene contents of 21 composite samples of canned apricots, 30 all green asparagus, 12 culturally bleached asparagus, 73 green beans, 26 lima beans, 5 baked beans, 27 beets, 19 carrots, 61 yellow corn, 14 orange juice, 17 clingstone peaches, 13 freestone peaches, 98 peas, 17 sliced pineapple, 18 pineapple juice, 10 prunes, 31 spinach, 61 tomatoes, and 79 tomato juice have been determined. The ascorbic acid content of 45 samples of white corn, 22 grapefruit segments, 43 grapefruit juice, and 30 pears, and the carotene content of 28 samples of white corn, 3 grapefruit segments, 18 grapefruit juice, and 4 pears have also been determined.

Eight composite samples of mackerel, 5 salmon, 15 sardines, 8 shrimp, and 6 tuna have been analyzed for vitamin A.

The analytical data for each food have been presented in tabular form as range and average values.

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# THE NUTRITIVE VALUE OF CANNED FOODS

## III. THIAMINE AND NIACIN<sup>1</sup>

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In an effort to enlarge upon present knowledge of the nutritional value of canned foods the National Cannery Association-Can Manufacturers Institute has collected a large number of canned food samples and has distributed these to university laboratories for assay. It is the purpose of this paper to report the results of thiamine and niacin assays on these samples.

### EXPERIMENTAL

The collection and distribution of the samples is described in a separate paper (Clifcorn, '44). The same report contains the details of coding, packing dates, collection by areas and can sizes. When received at this laboratory the coded samples were stored in the basement room until assayed. The temperature of the room ranged from 22°C. to 26°C. for the duration of the work, September 1942 to August 1943.

#### *Preparation of samples*

The contents of all cans taken for one sample (6 consumer size cans or 1 to 3 no. 10 cans) were combined and mixed. The solids of green beans, lima beans, carrots, white and yellow whole kernel corn, peas, apricots, asparagus, beets, grapefruit segments, peaches, tomatoes, pineapple slices, spinach, and pears were separated from the liquids on a screen. The solids were weighed and the liquid volume determined. One-tenth of the solid and liquid portions (300 to 400 gm. total weight) were recombined, and this reconstituted aliquot was blended with 2-3 ml. of chloroform in a Waring Blendor. In the case of apricots,

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asparagus, beets, grapefruit segments, peaches, tomatoes, pineapple slices, spinach, and pears, the foods were macerated by a propeller-mixer or cut to a small particle size by hand before the solid and liquid portions were separated. Prunes were treated in a similar manner, except that the pits were removed and only the edible portion macerated, blended and analyzed.

The various canned juices (tomato, grapefruit, orange and pineapple) were not homogenized in the Waring Blendor, but the contents of the individual cans were simply stirred together. The desired aliquot was then removed and 2-3 ml. of chloroform added.

Baked beans, salmon, dry-packed shrimp, and sardines in tomato sauce were hand-mixed, and a weighed amount, plus 2-3 ml. of chloroform, was blended with sufficient water to form a thick slurry. Tuna, brine-packed shrimp, sardines in oil, and brine-packed mackerel, however, were drained, the oil or brine discarded, and the solid portion then treated in the same way as the baked beans, salmon, shrimp and sardines.

#### *Assay methods*

*Thiamine.* Ten-gram samples of the blended foods were weighed out for extraction with 0.1 N  $\text{H}_2\text{SO}_4$  and subsequent analysis. Thiamine was determined by the thiochrome method of the Research Corporation Committee based on the thiochrome method (Hennessy, '42) with the following modifications: (a) extractions were made at 70°C. for 1 hour rather than  $\frac{1}{2}$  hour in boiling water; (b) enzymatic digestion was performed at 37°C. over-night rather than 2 hours at 45-50°C.; (c) instead of using a separate series of standards, a standard value was obtained by adding a standard thiamine solution to a third aliquot of the KCl-HCl eluates and determining the increased thiochrome produced over that of the eluate alone.

*Niacin.* Five grams of the homogenized sample were hydrolyzed with 50 ml. of N NaOH at 15 pounds pressure for 30 minutes. After hydrolysis the suspension was adjusted to a pH of 6.6-6.8 with N HCl (brom thymol blue, outside indicator) and diluted to give an estimated concentration of 0.05 to 0.1  $\mu\text{g}$ . of niacin per milliliter. Fish samples were hydrolyzed with 50 ml. of N  $\text{H}_2\text{SO}_4$  at 15 pounds pressure for 30 minutes. The sample was then adjusted to a pH of 6.6-6.8 with N NaOH and diluted to the same concentration as mentioned above.

The assay method of Snell and Wright ('41) was employed with the modified medium of Krehl, Strong and Elvehjem ('43). Each sample was set up at five levels (1, 2, 3, 4, and 5 ml. of test suspension per tube), and duplicate assays were run on different days.

## RESULTS AND DISCUSSION

The results obtained are summarized in table 1. The values are expressed as milligrams of the vitamin per 100 gm. of the entire original contents of the can except in the cases noted previously. The data presented are believed to be much more extensive than anything heretofore published on the thiamine and niacin content of canned foods. Sufficiently large numbers of samples were analyzed so that the average values found may be considered to represent quite closely the true vitamin content of the foods studied.

TABLE 1  
*Thiamine and niacin content of canned foods.*

PRODUCT	NO OF SAMPLES	THIAMINE HCl		NIACIN	
		Range	Average	Range	Average
		mg %	mg %	mg. %	mg. %
Apricots, unpeeled halves	21	0.003-0.026	0.019	0.20-0.48	0.37
Asparagus, all green	30	0.040-0.122	0.067	0.22-1.19	0.85
Culturally bleached	12	0.043-0.055	0.050	0.63-0.88	0.75
Beans, baked New England style	3	0.015-0.027	0.021	0.15-0.32	0.24
With tomato sauce	2		0.053		0.91
Green cut	75	0.011-0.053	0.029	0.18-0.60	0.32
Lima, green	27	0.019-0.048	0.032	0.32-0.77	0.55
Beets	27	0.004-0.014	0.008	0.06-0.28	0.13
Carrots	19	0.013-0.027	0.021	0.19-0.49	0.34
Corn, white, whole kernel	46	0.010-0.039	0.021	0.46-1.50	0.88
Yellow, whole kernel	61	0.014-0.045	0.026	0.53-1.06	0.77
Grapefruit juice	43	0.012-0.050	0.025	0.08-0.49	0.17
Segments	24	0.007-0.046	0.026	0.13-0.35	0.21
Mackerel	9	0.021-0.045	0.034	4.01-11.4	7.82
Orange juice	13	0.033-0.103	0.072	0.18-0.30	0.25
Peaches, clingstone, halves	17	0.005-0.011	0.007	0.48-1.18	0.70
Freestone, halves	13	0.005-0.011	0.008	0.34-0.90	0.57
Pears, halves	30	0.005-0.012	0.009	0.06-0.28	0.13
Peas, sweet, wrinkled varieties	94	0.056-0.183	0.115	0.42-2.69	1.06
Alaska	6	0.085-0.121	0.099	0.68-1.04	0.80
Pineapple juice	18	0.031-0.070	0.052	0.16-0.20	0.18
Sliced	17	0.053-0.087	0.070	0.12-0.20	0.17
Prunes, Italian	10	0.017-0.039	0.024	0.19-0.47	0.36
Salmon	5	0.014-0.038	0.021	5.95-8.91	7.81
Sardines, in oil	5	0.014-0.042	0.024	2.92-7.15	5.57
In tomato sauce	10	0.007-0.016	0.010	2.36-5.40	3.93
Shrimp, dry pack	3	0.006-0.011	0.009	1.10-3.40	2.23
Wet pack	5	0.004-0.011	0.008	0.72-2.52	1.36
Spinach	31	0.009-0.041	0.020	0.16-0.64	0.30
Tomatoes	63	0.019-0.077	0.049	0.41-0.97	0.69
Tomato juice	77	0.014-0.063	0.049	0.55-1.77	0.75
Tuna	6	0.016-0.082	0.037	7.60-13.0	10.2

The extent of the range in thiamine content varied considerably from product to product. The highest values for apricots, green beans, white corn, grapefruit juice, peas, spinach, tomato juice and tomatoes were approximately four times the lowest values. The range for grapefruit sections and tuna fish was 5- to 6-fold, while that for the remaining foods was 3-fold or less.

TABLE 2  
*Comparison of can size and vitamin content.*

PRODUCT	CAN SIZE	NO. OF SAMPLES	THIAMINE		NIACIN	
			Range	Average	Range	Average
			mg. %	mg. %	mg. %	mg. %
Asparagus, all green	C <sup>1</sup>	20	0.045-0.122	0.071	0.60-1.07	0.89
	I <sup>2</sup>	10	0.040-0.090	0.059	0.22-1.19	0.78
Culturally bleached	C	8	0.044-0.055	0.050	0.64-0.83	0.74
	I	4	0.043-0.052	0.050	0.63-0.88	0.77
Beans, green cut	C	54	0.011-0.053	0.028	0.18-0.60	0.31
	I	21	0.017-0.047	0.031	0.27-0.44	0.32
Lima, green	C	18	0.023-0.048	0.034	0.32-0.77	0.53
	I	9	0.019-0.041	0.027	0.33-0.67	0.58
Beets	C	17	0.004-0.012	0.008	0.06-0.28	0.12
	I	10	0.006-0.014	0.009	0.08-0.22	0.14
Carrots	C	12	0.013-0.027	0.021	0.25-0.49	0.35
	I	7	0.017-0.025	0.022	0.19-0.45	0.32
Corn						
white, whole kernel	C	40	0.010-0.039	0.022	0.46-1.50	0.83
	I	6	0.010-0.023	0.017	0.86-1.04	0.90
Yellow, whole kernel	C	47	0.015-0.045	0.027	0.56-0.95	0.75
	I	14	0.014-0.037	0.021	0.53-1.06	0.81
Peas	C	80	0.056-0.188	0.119	0.62-2.69	1.10
	I	20	0.078-0.146	0.103	0.42-1.34	0.96
Spinach	C	20	0.010-0.041	0.021	0.20-0.64	0.32
	I	11	0.009-0.025	0.018	0.16-0.51	0.27
Tomatoes	C	58	0.019-0.061	0.047	0.41-0.97	0.68
	I	5	0.045-0.077	0.063	0.74-0.96	0.86

<sup>1</sup> C = consumer size can.

<sup>2</sup> I = institutional size can.

The thiamine content did not seem to be correlated with the time of season except in the case of sweet peas. Early season peas were found to be slightly lower in thiamine content than mid-season and late-season samples. Average values from seventeen factories were 14.1% higher at mid-season and 11.5% higher at late season than the early season samples. It should be noted that because of the relatively high thiamine content of peas such differences are more readily detected than in the other products. The pH of the liquid in the can did not seem to be a factor in the variations found in the thiamine content of any one

product. It would seem that the longer sterilization times used for the larger institutional can sizes might reduce the thiamine content to lower values than those found in consumer size cans. The comparison in table 2, however, fails to show a consistent difference.

As expected, the fish products were found to contain more niacin than any other type of food analyzed. The values found ranged from 10.2 mg. % in tuna fish to 1.36 mg. % in shrimp. Of the canned vegetables analyzed, asparagus, corn, peas, and tomato juice were found to contribute the most niacin to the dietary, but were still relatively low in this vitamin since they contained only 0.75–1.06 mg. %. Fruits as a group were still lower, less than 0.7 mg. % being present.

As in the case of thiamine, there was no difference in niacin content of foods packed in the larger or smaller cans, as may be seen by comparing the average values in table 2. An attempt was made to correlate the niacin values found for each type of food with the time of harvest. In no case was any correlation found which would help to explain the wide ranges of some of the products. These ranges, as in the case of the thiamine assays, varied from 2- to 6-fold.

Although in the case of most foods the range is rather wide, this wide range is due to only a few samples. Thus the niacin content of peas in over 88% of the samples analyzed fell within a 2-fold range, although the extreme range was about 6-fold.

#### SUMMARY

Average values and ranges for the thiamine and niacin content are reported for thirty-two canned food products. Considerable variation has been found. The thiamine content of peas appears to be slightly affected by the packing season. With this exception no correlation was found between thiamine or niacin content and can size, time of harvest, or pH of these products.

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# THE NUTRITIVE VALUE OF CANNED FOODS

## IV. RIBOFLAVIN AND PANTOTHENIC ACID<sup>1</sup>

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A more thorough knowledge of the vitamin content of canned foods is of obvious importance to those who formulate nutritional programs and dietaries. In an effort to obtain reliable data, the National Cannery Association—Can Manufacturers Institute, have collected and distributed samples of canned foods to a number of laboratories for assay. The method of collection, distribution, etc., has been described in detail in a previous report of this series (Clifton, '44). This laboratory has concerned itself with analyses of these samples for riboflavin and for pantothenic acid.

### EXPERIMENTAL

#### *Preparation of samples*

Detailed treatment of samples varied somewhat with the nature of the material assayed. In all cases, contents of all cans of a particular sample (usually six) were mixed. Ten grams of the mixed samples of juice (grapefruit, orange, pineapple, tomato) were then taken directly for enzyme treatment. With apricots, asparagus, beets, grapefruit segments, peaches, pears, pineapple, spinach and tomatoes, the entire sample was mixed in an earthenware jar with a propeller mixer until the state of subdivision was rather fine (about 5 minutes). Four hundred and fifty grams of this material were then transferred to a Waring Blendor, and mixed for 3 to 5 minutes longer until the material was thoroughly homogenized. A 10-gm. sample of this material was then removed for enzyme treatment. Baked beans, salmon, sardines in tomato sauce, and shrimp (dry-pack) were hand-mixed; 225 gm. were then transferred to the Blendor, 225 gm. of water added, the mixture blended as above, and 10 gm. of the blended material (equivalent to 5 gm. of the original sample) removed for enzyme treatment. With mack-

<sup>1</sup>This work was supported in part by a grant from the National Cannery Association—Can Manufacturers Institute Nutrition Program to Professor R. J. Williams (see Clifton, '44).

erel, sardines in oil, shrimp (wet-pack) and tuna, the liquid content of each can was discarded and the samples then mixed and treated in the same manner as the fish products above. With green beans, lima beans, carrots (diced), white and yellow corn, and peas, the liquid was drained off, and liquid and solid weights obtained. The solids were hand-mixed, a 450-gm. sample was reconstituted from the liquids and solids, and transferred to the Blendor, homogenized 3-5 minutes, and then a 10-gm. sample removed for enzyme treatment. Prunes were treated in the same manner as corn and peas, after preliminary removal of the pits.

For enzyme treatment, solutions of clarase and papain (Caroid) were prepared which contained 20 mg. of the enzyme dissolved (or uniformly suspended) in 1 ml. of acetate buffer, pH 4.5 (Cheldelin et al., '42). Five milliliters of each enzyme solution (equivalent to 100 mg. of each enzyme) were added to each 10-gm. sample of foodstuff. The mixture was diluted to 30 ml. with acetate buffer, and incubated for 24 hours at 37°C. under about 2 ml. of benzene. After incubation, the samples were heated at 100°C. for 15 minutes in flowing steam in order to remove benzene and precipitate proteins and then were diluted to 50 ml. with water. A small amount of Hyflo Supercel was added, and the samples then filtered with gentle suction. Assays for riboflavin and pantothenic acid were made on the filtrates. "Blank determinations" of the riboflavin and pantothenic acid concentrations in the enzyme samples were made by the same procedure at frequent intervals; this correction was subtracted from the values obtained on the sample. This correction varied slightly with different lots of enzyme, but averaged .032 mg. calcium pantothenate and .012 mg. riboflavin per 100 gm. of sample.

#### *Assay methods*

Riboflavin was determined by the method of Snell and Strong ('39); pantothenic acid was determined by the method of Pennington, Snell and Williams ('40). All assays were carried out on clear, filtered, enzymatic digests of the food sample, prepared as described above. Filtration was carried out at pH 4.5-4.7, and thus the presence of fatty materials which interfere in assays involving use of *L. casei* (Strong and Carpenter, '42; Wegner et al., '42) was avoided.

In general, the samples were satisfactory for the riboflavin assay. With the pantothenic acid assay, there was in many instances a drift in the values obtained. When this was present, those values calculated from low assay levels were considerably higher than those calculated

from higher levels. The values obtained tended to become constant at the higher assay levels (lowest assay figures). It was first attempted to remedy this situation by applying to the samples the method of Strong, Feeney and Earle ('41) and that of Landy and Dieken ('42). However, the same tendency was present to about the same degree when these methods were used. For purposes of assay the drift was therefore allowed to occur, but when it occurred only the lower values were used. These were obtained on the upper portion of the standard curve, where the drift in values was largely eliminated. Recovery experiments with added pantothenic acid showed 100% recovery at these levels, indicating validity of the results. Previous experience with microbiological assay methods has indicated that when such a drift occurs, the lower values, obtained at higher assay levels, approach more closely the true vitamin content of the material in question. Toward the end of the assay program, a modified method for pantothenic acid appeared<sup>2</sup> (Neal and Strong, '43). This method was sensitive to smaller amounts of pantothenic acid, and also largely eliminated drift in samples where this was apparent. Numerous assays were therefore made according to this procedure. Excellent agreement between results obtained by this method and the method used throughout the present investigation indicates reliability of results obtained with either technique (cf. table 2).

#### RESULTS AND DISCUSSION

A summary of the assay results obtained is given in table 1. Comparative assay results obtained by the method of Neal and Strong ('43) and that of Pennington, Snell and Williams ('40) are given in table 2.

One of the most interesting facts to emerge from these assays is the great variation in vitamin content which occurs from one sample to another of any one foodstuff. Examination of table 1 reveals that different samples of most products vary 3- to 4-fold in their vitamin contents; in some cases even greater variation is evident. This variation becomes more significant when it is remembered that most of the samples assayed consisted of the pooled contents of six cans of material. It emphasizes the unsatisfactory nature of conclusions based upon assays of the contents of individual cans, picked at random from shelves of retailers. The range as given in the table, however, is somewhat misleading, since it represents the extreme variations which occurred. Assays on the great majority of samples lay much closer to the average than these extremes would indicate.

<sup>2</sup> The authors wish to thank Dr. Strong for use of his manuscript in advance of publication.



Information supplied with the samples permitted their classification according to can size and packing date—early, late, or mid-season. Where the number of samples justified it, classification was made on each of the above bases. No significant differences in average values obtained appeared in any case.

TABLE 1  
*Pantothenic acid and riboflavin content of canned foods.*

PRODUCT	NO. OF SAMPLES		CALCIUM PANTOTHENATE		RIBOFLAVIN	
	Retail size cans	cans No. 10	Range	Average	Range	Average
			mg./100 gm.	mg./100 gm.	mg./100 gm.	mg./100 gm.
Apricots, unpeeled halves	21		.045-.20	.095	.012-.039	.024
Asparagus, all green	20	11	.072-.43	.19	.039-.19	.096
Culturally bleached	8	4	.092-.16	.12	.044-.073	.058
Beans, baked						
New England style		3	.061-.11	.081	.051-.059	.054
With tomato sauce		2	.092-.11	.10	.019-.029	.024
Green cut	53	19	.023-.11	.061	.018-.065	.034
Lima, green	17	9	.068-.17	.11	.023-.062	.042
Beets	18	9	.045-.12	.076	.011-.059	.025
Carrots	12	7	.093-.21	.13	.009-.042	.021
Corn, white, whole kernel	40	6	.10-.50	.18	.027-.064	.043
Yellow, whole kernel	47	14	.094-.32	.21	.025-.072	.044
Grapefruit juice	43		.065-.17	.12	.010-.033	.019
Segments	24		.063-.19	.13	.008-.039	.021
Mackerel	9		.13-.48	.29	.13-.29	.20
Orange juice	15		.081-.17	.12	.012-.038	.022
Peaches, clingstone, halves	17		.017-.065	.041	.013-.030	.022
Freestone, halves	14		.020-.13	.052	.009-.034	.021
Pears, halves	30		.008-.041	.022	.009-.032	.019
Peas, sweet, wrinkled						
varieties	73	21	.081-.26	.15	.025-.10	.054
Alaska	6	1	.069-.18	.13	.037-.064	.049
Pineapple juice	18		.066-.18	.10	.004-.031	.018
Sliced	17		.053-.15	.10	.006-.030	.021
Prunes, Italian <sup>1</sup>	10		.027-.085	.044	.019-.032	.026
Salmon	5		.47-.73	.57	.14-.17	.16
Sardines, in oil	5		.44-.65	.53	.09-.15	.11
In tomato sauce	10		.41-.58	.47	.12-.23	.18
Shrimp, dry pack	3		.26-.35	.29	.027-.037	.032
Wet pack	5		.18-.22	.21	.026-.035	.031
Spinach	20	11	.021-.14	.051	.024-.13	.032
Tomatoes	58	5	.11-.44	.23	.011-.050	.028
Tomato juice	79		.17-.39	.25	.009-.046	.028
Tuna	6		.13-.19	.17	.11-.17	.14

<sup>1</sup> Reported on pit-free basis. The pitted samples weighed on the average 95% as much as the whole sample.

It is well known that pantothenic acid occurs naturally in forms which are unavailable for test organisms used in microbiological assays. For this reason, enzymatic digestion of samples is now universally employed to liberate additional pantothenic acid. The enzymatic digestion procedure used throughout this work was that of Cheldelin et al. ('42). Application of this method to animal products results in large increases in pantothenic acid over those obtained by water extraction. At the time this assay program was undertaken, no investigation of the efficiency of enzymatic digestion procedures in liberating

TABLE 2  
*Comparative assays for pantothenic acid.*

PRODUCT	NO OF SAMPLES	CALCIUM PANTOTHENATE			
		Pennington et al.		Neal and Strong	
		Range	Average	Range	Average
		mg./100 gm.	mg./100 gm.	mg./100 gm.	mg./100 gm.
Beans, green cut	6	.060-.075	.065	.059-.090	.066
Green lima	3	.11-.13	.12	.06-.14	.10
Beets	1		.071		.070
Carrots	1		.13		.14
Corn, white, whole kernel	7	.16-.50	.26	.16-.42	.27
Yellow, whole kernel	13	.20-.32	.23	.19-.30	.26
Pears	1		.017		.019
Peas,					
sweet, wrinkled varieties	4	.13-.15	.14	.06-.20	.14.
Pineapple, sliced	1		.03		.10
Sardines, in oil	1		.49		.56
Shrimp	4	.20-.26	.22	.20-.29	.22
Spinach	1		.039		.033
Tomatoes	1		.23		.30
Tomato juice	2		.27		.27
Tuna	2		.19		.19

pantothenic acid from cooked plant or animal tissues had been made. It is possible that other digestion procedures would liberate more pantothenic acid than that indicated by our assays. The figures obtained here for pantothenic acid should thus be regarded as minimum values for the canned product in question.

The use of enzymatic digestion preliminary to riboflavin assay is not so common. For the above program, use of such a procedure presented the very considerable advantage that the preparation of only one extract instead of two was necessary. Preliminary trials with a number of samples showed that values obtained by the above procedure checked those obtained by the more commonly used procedures of autoclaving

with water (Snell and Strong, '39) or with dilute acid (e. g. Wegner et al., '42). Cheldelin et al. ('42) also found enzymatic digestion to be as efficient as neutral- or acid-autoclaving in liberating riboflavin, while digestion with pepsin was recommended by Van Duyne ('41) for the extraction of riboflavin from tissues.

With both pantothenic acid and riboflavin, the correction in assay value rendered necessary by the vitamin content of the enzyme preparation used is considerable; in some cases it approaches in magnitude the vitamin content of the product in question. Values for this correction are entirely reproducible, however, so that the procedure would lead to error only if the value for the enzyme correction considerably surpassed the assay value of the sample. This situation did not exist. That this factor did not influence the accuracy of the assays is shown by the agreement of values obtained by two methods for pantothenic acid (table 2), and the above-mentioned agreement in assay values for riboflavin obtained after enzyme-treatment, and after extraction with acid.

#### SUMMARY

Average values for the riboflavin and pantothenic acid content of 32 different types of canned foods are given. The amount of these vitamins present in different samples of any given canned food varies considerably. Reasons for this variation are not apparent from the present study. It was not correlated with can-size, or with the time during the growing season that the food was packed.

#### ACKNOWLEDGMENT

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# THE NUTRITIVE VALUE OF CANNED FOODS

## V. DISTRIBUTION OF WATER SOLUBLE VITAMINS BETWEEN SOLID AND LIQUID PORTIONS OF CANNED VEGETABLES AND FRUITS

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This portion of the research program of the National Canners Association—Can Manufacturers Institute was designed to supplement the large body of data on vitamin contents of a great variety of canned foods to be obtained by other laboratories, with information about the distribution of water-soluble vitamins in a representative number of canned vegetables and fruits. A fair picture of such distribution could be obtained, it was thought, by determination of three of these vitamins; namely, ascorbic acid, thiamine, and riboflavin in the solid and liquid portions of several types of the canned products. Thus the proportions of the three vitamins found in the solid and in the brine of eight different canned vegetables, both in consumer size and in no. 10 size cans, as well as those found in the solid and in the syrup of seven canned fruits, in consumer size cans only, are here reported.

### EXPERIMENTAL METHODS

#### *Separation and weighing of solid and liquid*

Separation of solid and liquid portions for distribution studies was carried out approximately according to the standard techniques described by the Association of Official Agricultural Chemists. The only variations from these procedures were that a 12-inch sieve, rather than an 8-inch one, was used for drainage of whole single no. 2 or no. 2½ cans, and that for all vegetables in no. 10 cans, except spinach, the contents of each can were drained in two portions, each for 2 minutes, on the 12-inch sieve. For no. 10 cans of spinach the contents of a can were drained in three portions, each for 3 minutes.

The weights of solid and liquid recorded in the tables on the "per can" basis are, in the case of consumer sizes, the single can averages of the weights obtained on mixtures of solids and mixtures of liquids combined from three or six cans, and are, in the case of no. 10 cans, the averages of six separate weighings of the two portions of six no. 10 cans.

### *Sampling for analyses*

For vegetables in consumer size cans, the whole amount of solids from three (or six) cans was mixed as well as possible without macerating the material and then two approximately 150- to 200-gm. samples were removed from the mixture and separately blended in the Waring Blendor with measured amounts of 3% metaphosphoric acid and 0.1 N sulfuric acid in preparation for the extraction of ascorbic acid and thiamine-riboflavin respectively. The whole lot of solid from three such cans was deliberately not more intimately mixed, as by maceration, before the samples were withdrawn, because, for the other part of this study, the remaining two can quantities of solid were required intact. These were to be further divided for reconstituting with liquid to form representative single can contents which would be used in duplicate experiments on vitamin retentions in heating as in family scale preparation for serving.

For vegetables in no. 10 cans, 200-gm. samples were removed from each can's solid portion before the remainders were combined for the experiments on large scale preparation for serving. The 1200 gm. thus brought together from six cans were thoroughly mixed with a beater in the "Kitchen Aid" bowl and the 200-gm. samples then used for vitamin determinations were taken into the Waring Blendor cup from this macerated mixture.

For fruits, no heating experiments were carried out and since in these the solid portions were all large pieces, the mixture of solids from six consumer size cans were necessarily also macerated in the "Kitchen Aid" bowl before samples were taken for analyses.

Sampling of solid portions was further continued by transferring appropriate aliquots of blends, to volumetric flasks for buffering in the case of ascorbic acid determination, and to 250-ml. centrifuge bottles for hot extraction with 0.1 N sulfuric acid in the case of thiamine and riboflavin determinations.

In sampling the liquid, the weighed portions were withdrawn from the complete mixture of liquid from all the cans of a set, and were transferred to the appropriate acids. For ascorbic acid extracts, 25- to 50-gm. samples were taken and for thiamine-riboflavin digests, 15- to 30-gm. samples.

### *Methods of analyses*

Chemical methods of analyses were employed for determination of the three vitamins.

For most of the ascorbic acid determinations, the Morell ('41) modification of the Bessey ('38) method for photoelectric measurement of the rednctions of the indophenol dye was used, employing the Coleman Universal Spectrophotometer Model II. For these, the wave length dial was set at 500 m $\mu$ , since that was found to be the wave length of maximum absorption for the partieuclar batch of dye used throughout the experiments. The calibration equation derived by the method of least squares was used for caleulations, rather than a plotted curve. However, even with the use of such an equation, dilutions of ascorbic acid in the final test solution below 0.002 mg, per milliliter were avoided, since determinations below this level were apt to have errors in excess of 10%.

In the first four experiments, namely, those on sweet peas in table 1, the Bessey ('38) technique was used, with the wave length dial set at 520 m $\mu$ . In these experiments, to avoid unduly large errors, the concentration of ascorbic acid in the final test solution was not allowed to fall below 0.004 mg. per milliliter.

Finally, because the Spectrophotometer had to be sent away for a galvanometer repair, it was necessary to use the titration method in a considerable number of determinations averaged in table 1; namely, those on green beans, all of those on corn, and in one on spinach. In these, a 3% metaphosphoric acid extract was titrated to a 15-second end-point with the dye.

For thiamine and riboflavin determinations, acid digestion was followed by overnight incubation with 0.4% clarase at 37°C. and ph 4.3 to 4.5. Then the double adsorption technique of Conner and Straub ('41) was used.

Since we have had some difficulty regarding satisfactory recovery of the vitamins from recent samples of their respective adsorbents and have learned that others have likewise had trouble, it would seem to be appropriate to state here that all samples of Decalso and Florisil employed in this study had been tested with solutions of crystalline thiamine and riboflavin respectively, and that no adsorbent was put into use which gave less than 93% recovery. Moreover, with each set of determinations on a vegetable or fruit, one recovery experiment was included, in which synthetic thiamine and riboflavin were added to a digestion bottle along with a third aliquot of the blend of the solid sample and carried through the whole procedure. Among forty-eight such determinations, thiamine recoveries ranged from 90 to 107% in forty-one of them and were 75, 75, 84, 109, 113, and 116% in the remaining seven, whereas riboflavin recoveries ranged from 85 to 106%



TABLE 1  
Distribution of water soluble vitamins in consumer size cans of vegetables.<sup>1</sup>

VEGETABLE	NO. OF EXPERI- MENTS IN AVERAGES	WEIGHT		ASCORBIC ACID		THIAMINE		RIBOFLAVIN	
		Total per can	Distribu- tion	Concen- tration	Distribu- tion	Concen- tration	Distribu- tion	Concen- tration	Distribu- tion
		gm.	%	mg./100 gm.	%	mg./100 gm.	%	mg./100 gm.	%
Asparagus, all green	1	Solid	62	17.6	60	0.065	62	0.124	71
		Liquid	38	10.2	40	0.065	38	0.081	29
Beans, green cut	5 <sup>2</sup>	Solid	64	5.41	64	0.036	67	0.054	76
		Liquid	36	5.54	36	0.032	33	0.030	24
Beans, lima, green	4 <sup>3</sup>	Solid	70	5.91	56	0.028	68	0.054	76
		Liquid	30	10.3	44	0.030	32	0.039	24
Carrots	1	Solid	64	2.55	66	0.022	66	0.021	74
		Liquid	36	1.97	34	0.020	34	0.013	26
Corn, white whole kernel	1	Solid	67	3.86	48	0.015	67	0.031	80
		Liquid	33	8.54	52	0.015	33	0.026	20
Corn, yellow whole kernel	4 <sup>4</sup>	Solid	68	5.19	61	0.034	67	0.063	78
		Liquid	32	7.18	39	0.035	33	0.036	22
Peas, sweet wrinkled varieties	4 <sup>5</sup>	Solid	66	9.30	63	0.120	66	0.065	70
		Liquid	34	10.6	37	0.121	34	0.054	30
Spinach	2 <sup>6</sup>	Solid	63	5.93	62	0.013	69	0.080	76
		Liquid	37	6.15	38	0.014	31	0.042	24

<sup>1</sup> For asparagus, carrots, and white corn, six no. 2 cans were mixed; for each experiment with green beans, lima beans, yellow corn, and sweet peas, three no. 2 cans were mixed; and for each experiment with spinach, six no. 2½ cans.

<sup>2</sup> Except for ascorbic acid, 4 experiments.

<sup>3</sup> Except for ascorbic acid, 2 experiments.

<sup>4</sup> Except for thiamine, 3 experiments, and for riboflavin, 2 experiments.

<sup>5</sup> Except for thiamine, 3 experiments.

<sup>6</sup> Except for thiamine, 1 experiment.

TABLE 2  
Distribution of water soluble vitamins in no. 10 cans of vegetables.<sup>1</sup>

VEGETABLE		VITAMIN C		ASCORBIC ACID		THIAMINE		RIBOFLAVIN	
		Total per can	Distribution	Concentration	Distribution	Concentration	Distribution	Concentration	Distribution
		gm.	%	mg./100 gm.	%	mg./100 gm.	%	mg./100 gm.	%
Asparagus, all green	Solid Liquid	1898	62	13.1	63	0.077	62	0.114	69
		1147	38	12.8	37	0.077	38	0.086	31
Beans, green cut	Solid Liquid	1848	61	2.35	61	0.028	67	0.040	74
		1183	39	2.37	39	0.022	33	0.023	26
Beans, lima, green	Solid Liquid	2309	71	7.02	58	0.020	67	0.055	76
		942	29	12.5	42	0.025	33	0.014	24
Carrots	Solid Liquid	2203	73	1.28	68	0.022	72	0.024	78
		846	27	1.61	32	0.022	28	0.018	22
Corn, white whole kernel	Solid Liquid	2013	64	4.70	54	0.010	61	0.051	76
		1148	36	6.93	46	0.011	39	0.029	24
Corn, yellow whole kernel	Solid Liquid	1985	64	2.09	46	0.012	63	0.062	73
		1103	36	4.36	54	0.012	37	0.044	28
Peas, sweet wrinkled varieties	Solid Liquid	2083	69	7.43	62	0.112	68	0.077	73
		970	31	10.00	38	0.118	32	0.062	27
Spinach	Solid Liquid	1497	51	16.2	47	0.024	53	0.132	60
		1428	49	19.0	53	0.022	47	0.094	40

<sup>1</sup> For all vegetables, six no. 10 cans were mixed in each experiment and each average value is calculated from two such experiments.

in forty out of forty-seven determinations and were 73, 82, 82, 110, 110, 111, and 116 in the remainder.

For fluorescence readings the Coleman Electronic Photofluorometer was used. For standardizing the instrument sensitivity the usual stable fluophor, quinine sulfate (0.135  $\mu\text{g.}$  per milliliter) in 0.1 N  $\text{H}_2\text{SO}_4$  was used for the thiochrome readings, whereas sodium fluorescein (0.100  $\mu\text{g.}$  per milliliter) in 0.01 N NaOH was used for riboflavin.

The most important of the variations made by us from the rest of the Conner and Straub procedure concerned the fluorescence determinations in the eluates of thiamine and riboflavin. For both, individual non-specific fluorescence blank readings were made; in the former, by the usual Hennessy and Cerecedo ('39) technique of omitting the potassium ferricyanide; in the latter, by exposing some of each eluate to light (Najjar, 41), using a General Electric AH-4 100-watt mercury vapor lamp in an ordinary pyrex test tube at a distance of 12 mm. from the lamp bulb for a period (22 to 30 min.) which in preliminary tests, had been determined as sufficient to destroy all the riboflavin, following which the same  $\text{KMnO}_4$  and  $\text{H}_2\text{O}_2$  treatment used on an unexposed aliquot of eluate was also given to the blank. Further appropriate corrections were also made on all readings for thiamine and riboflavin fluorescence values of those amounts of clarase represented in the final test aliquots. Moreover, for both vitamin determinations internal standard readings were taken as the basis for calculation, using the average of the increments in readings in a series, obtained by the addition of 0.2  $\mu\text{g.}$  (or 0.4) of thiamine and 1.0  $\mu\text{g.}$  (or 0.5) of riboflavin to one aliquot from each of the respective eluates.

## DISCUSSION OF RESULTS

### *Vegetables*

*Duplication of vitamin values in two lots from the same pack.* Vitamin concentration values in duplicate 3-can lots of consumer size cans and in duplicate 6-can lots of no. 10 cans of identical code numbers were in general, in good agreement for thiamine and riboflavin, but not so good for ascorbic acid in nos. 2 and 2½ cans and still less satisfactory for ascorbic acid in no. 10 cans.

For thiamine and riboflavin, the variations between such duplicate lots for all can sizes were not over 10% except in five cases out of twenty-four for each vitamin, these running between 11 and 21% for the thiamine and between 13 and 33% for riboflavin.

For ascorbic acid the variation in duplicate lots of nos. 2 and 2½ cans was over 20% in only one sample out of fourteen, whereas, in

no. 10 cans there were six instances out of a possible ten in which variations were over 20% and reached as high as 40%.

*Comparative concentrations of vitamins in solids and liquids (all can sizes).* The concentration of ascorbic acid in the solid ran generally somewhat lower than in the liquid, the differences being most pronounced (50% to 100% of the solid value) for such products as corn and lima beans, in which a relatively lower water content of the solid portion probably explains the exaggerated difference in the amount of ascorbic acid dissolved in the two phases.

The concentrations of thiamine in solid and liquid were the same, within experimental error, with eight exceptions among thirty-five possibilities. In the eight exceptional determinations, the variations between thiamine values for solids and liquids ran from 20% to 33%, and in both directions.

The concentration of riboflavin in the solid was considerably higher than in the liquid in all cases, running higher from about 15% to 20% of solid value in the case of the solid of peas and some lima beans, to 40% to 50% in the case of the solid of some samples of green beans, corn and spinach.

*Distribution of weight, ascorbic acid, thiamine and riboflavin between solid and liquid.* For all sizes of cans and all vegetables except spinach, and one lot of asparagus in no. 10 cans, the solid weight was in the range of 60% to 73%. One 6-can lot of no. 2½ cans of spinach had solid weight of 55% and two 6-can lots of no. 10 cans of spinach had solid weight of 48% and 54%, whereas the exceptional lot of asparagus contained 58% of solid.

For all sizes of cans: in asparagus, green beans, carrots and peas, the solid carried 60% to 68% of the ascorbic acid; whereas, in white and yellow corn, lima beans, and spinach, the solid carried 46% to 58% of the ascorbic acid. However, in consumer size cans there was one lot out of two, of yellow corn and spinach which fell in the range of the first group of vegetables in their ascorbic acid distribution.

For all sizes of cans and all vegetables, except some asparagus and corn and all spinach in no. 10 cans, the solid carried from 62% to 72% of the thiamine. In one out of two 6-can lots each of asparagus and white corn in no. 10 cans, the solid carried 58% and 59%, respectively, whereas for spinach in no. 10 cans the average was 53%.

For all sizes of cans, and all vegetables except spinach, the solid carried 70% to 80% of the riboflavin. Spinach varied a great deal, for in no. 2½ cans the solid had 69% and 82% of the riboflavin, whereas in no. 10 cans it varied from 57% to 62%. In one out of two lots of

TABLE 3  
*Distribution of water soluble vitamins in consumer size cans of fruits.<sup>1</sup>*

FRUIT		WEIGHT		ASCORBIC ACID		THIAMINE		RIBOFLAVIN	
		Total per can	Distribution	Concentration	Distribution	Concentration	Distribution	Concentration	Distribution
		gm.	%	mg./100 gm.	%	mg./100 gm.	%	mg./100 gm.	%
Apricots, unpeeled halves (ripe)	Solid	467	53*	3.25	53	0.020	50	0.012	65
	Liquid	419	47	3.21	47	0.022	50	0.011	45
Grapefruit segments	Solid	347	57	23.0	56	0.034	58	0.006	67
	Liquid	262	43	24.0	44	0.032	42	0.004	33
Peaches, clingstone	Solid	567	67	4.81	67	0.007	70	0.012	61
	Liquid	284	33	4.68	33	0.006	30	0.015	39
Peaches, freestone	Solid	431	51	2.42	49	0.006	61	0.011	56
	Liquid	417	49	2.64	51	0.004	39	0.009	44
Pears	Solid	518	61	1.18	68	0.006	57	0.011	69
	Liquid	329	39	0.86	32	0.007	43	0.008	31
Pineapple, sliced	Solid	523	60	4.76	59	0.077	60	0.004	66
	Liquid	352	40	4.89	41	0.078	40	0.003	34
Prunes, italian	Solid	383	46	0.96	49	0.022	51	0.012	53
	Liquid	456	54	0.85	51	0.018	49	0.009	47

<sup>1</sup> Six no. 2½ cans were mixed for all fruits except grapefruit for which six no. 2 cans were used. Each value is from a single experiment.

asparagus, in no. 10 cans, the solid also had less of the riboflavin, 64%.

### *Fruits*

*Comparative concentrations of vitamins in solids and liquids. (Consumer size cans only).* In contrast to vegetables, the ascorbic acid concentration in the solids of canned fruits was the same, within experimental error, as in the liquids except in the case of pears. For these the solid had a 20% higher value than the liquid.

As for vegetables, thiamine concentrations are about the same in solids and liquids, except possibly in prunes, in which the 20% higher value for solids should be significant, the difference being in excess of experimental errors.

Also as for vegetables, riboflavin values for solids are higher than those for liquids in all fruits except cling peaches. Although in most instances the percentage differences are as great as for vegetables, they are not as significant, especially for the lowest values, because of the large experimental errors in such low values.

*Distribution of weight, ascorbic acid, thiamine and riboflavin between solid and liquid.* The whole range of solid weight is 46% to 67%, with prunes and freestone peaches lowest and cling peaches highest. There are too few values to break them down into groups.

The total ascorbic acid carried by the solid follows quite closely the percentages of solid weights and therefore the values are also difficult to group. In pears, however, higher ascorbic acid concentration in the solid which has been noted, shows up in the percentage figure for ascorbic acid in the solid.

The total thiamine carried by the solid also follows the percentages of solid weight, being significantly higher only for prunes, as might be expected from the concentration values, and for freestone peaches, where the difference may be due to unduly large errors in the extremely low thiamine values.

The total riboflavin carried by the solid is by percentage 5% to 12% higher than the percentage of solid weight, except, of course, in cling peaches, where it is lower in accordance with its lower concentration in solid.

### SUMMARY

To obtain information about the distribution of water-soluble vitamins in canned vegetables and fruits, determinations of ascorbic acid, thiamine and riboflavin were made on the drained solids and liquids of mixed 3-can or mixed 6-can lots of identical pack, including eight dif-

ferent vegetables, both in consumer size and in no. 10 size cans, and seven different fruits in consumer size cans. The degree of agreement between the concentrations of these vitamins in duplicate lots of identical pack and the comparison of the concentrations in solids and liquids are summarized. Regarding the over-all distributions, the observations led to the following general conclusions: (1) in most canned vegetables the solid weight, being 60% to 73% of the total can contents, carried 46% to 68% of the ascorbic acid, 62% to 72% of the thiamine, and 70% to 80% of the riboflavin. Spinach was the outstanding exception to these ranges, for with solid weights ranging from 48% to 55% of the total, all vitamin contents of the solid were correspondingly lower. (2) In fruits, the solid weights of the packs showed more variation, being 46% to 67%, and the vitamin percentages borne by these solids were consequently also more variable, ascorbic acid and thiamine percentages in solid agreeing closely with the weight percentages, and riboflavin paralleling them at about 5% to 12% higher level.

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# EFFECT OF THE COMPOSITION OF THE DIET ON THE RIBOFLAVIN REQUIREMENT OF THE RAT<sup>1</sup>

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(Received for publication April 10, 1944)

In a previous report (Mannering, Lipton and Elvehjem, '41) data were presented to show that the fat content of the diet had a marked effect on the riboflavin requirement of the rat. The feeding of riboflavin-low dextrin rations in which a large proportion of the carbohydrate had been isocalorically replaced by fat increased the amount of riboflavin needed by rats for growth and enabled the production of deficiency symptoms of greater severity than are usually observed. Animals fed these high fat rations developed a spastic paralysis of the hind quarters which could be prevented or cured by the administration of riboflavin. Shaw and Phillips ('41) have substantiated the above work and have been able to correlate the paralysis with degenerative neural changes.

The increased riboflavin requirement resulting from the substitution of fat for dextrin might be explained on the basis that an increased cellular demand for the vitamin occurs during the metabolism of fat, or, as seems more likely from the experiments that are to follow, that such an alteration in dietary constitution results in a decreased synthesis of available riboflavin by intestinal bacteria. Several papers have appeared in the literature emphasizing the role of dextrin in promoting the intestinal synthesis of certain vitamins. The coprophagy studies of Guerrant, Dutcher and Tomey ('35) and Guerrant, Dutcher and Brown ('37) demonstrated that the feces of dextrin-fed rats contained greater quantities of undifferentiated B complex than the feces from rats fed sucrose, lactose, glucose, or cornstarch. Guerrant and Dutcher ('34) studied the effects of various dietary constituents on the requirement of rats for thiamine and vitamin G (the heat stable portion of the

<sup>1</sup>Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from Commercial Solvents Corporation, and the Research Funds of the University.

We are indebted to Merck and Company, Rahway, New Jersey, for the synthetic vitamins; to Abbott Laboratories, North Chicago, Illinois, for haliver oil; and to Wilson Laboratories, Chicago, Illinois, for liver extracts.

<sup>2</sup>Rockefeller Fellow. Present address, Sao Paulo Medical School, Sao Paulo, Brazil.

B complex). They concluded that fat and protein had no effect, that higher levels of salts increased, and that agar, cellulose and dextrin decreased the amount of vitamin G needed in the diet. Although riboflavin was not considered independently of vitamin G, these studies strongly suggest that the amount of riboflavin required in the diet by the rat may depend in part on the amount of riboflavin supplied from an enteric source, and that this, in turn, can be greatly influenced by certain dietary ingredients, particularly dextrinized cornstarch.

The present investigation was undertaken to obtain further information regarding the relationship between dietary fat, dextrin, and riboflavin, and was later extended to include a study of the effect of other carbohydrates and of fat on the requirement for and fecal excretion of riboflavin.

#### EXPERIMENTAL AND RESULTS

##### *Effect of substituting fat for dextrin*

The composition of the riboflavin-low diets used is presented in table 1. Rations K-25 and K-27, containing 25 and 40% of fat, respectively, are modifications of high carbohydrate ration K-24 made by isodynamically replacing dextrin with lard. Ration K-26 differs from ration K-25 only in that a fat of vegetable origin<sup>3</sup> was substituted for lard. All rations used in this series and those following were kept under refrigeration. The dextrin was prepared by making a paste of commercial cornstarch and water, autoclaving for 3 hours at 15 pounds pressure, drying, and grinding.

Three-week-old male albino rats weighing 35 to 40 gm. were partially depleted of their riboflavin reserves for 2 weeks on ration K-24 before being divided into four groups of twelve animals receiving the above diets and varying amounts of riboflavin (0, 3, 6, 9, and 12  $\mu$ g. per day). The riboflavin was fed in  $\frac{1}{2}$  ml. of a 25% ethanol solution which was pipetted into small dishes. The animals were housed in individual cages having  $\frac{1}{2}$  inch mesh screen bottoms. Food consumption records were kept throughout the supplementation period. Spilled food was collected on paper towels placed beneath the cages and weighed. The results obtained during the 7 weeks following the depletion period are summarized in table 2.

Examination of the table reveals that at each level of riboflavin intake the growth of the animals receiving the high dextrin ration (7% fat) was superior to that of rats fed the 40% fat diet. The growth of animals fed the 25% fat rations was intermediate. No important dif-

<sup>3</sup> Crisco.

TABLE 1  
*Riboflavin-low rations.*

CONSTITUENTS	K-24	K-25	K-26	K-27	K-28	K-29	K-30*	K-31*	K-32*	K-33*	K-34*	K-35*	K-36*	K-37*	K-38*	K-39*
Dextrin, gm.	71	37.5	37.5	21.5	71	21.5	73	22.5	73	22.5	33		73	22.5		
Sucrose, gm.											40	22.5			73	
Lactose, gm.																
Cornstarch, gm.																
Extracted dextrin, <sup>1</sup> gm.																
Cellulose, <sup>2</sup> gm.		14											10	10		73
Lard, gm.																
Crisco, gm.			14													
Corn oil, gm.	2	2	2	2	2	2	5	5	5	22.5	5	5	5	5	5	5
Washed butterfat, <sup>3</sup> gm.	3	3	3	3	3	3										
Cod liver oil, gm.	2	2	2	2	2	2										
Caseln, <sup>4</sup> gm.	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
Salts IV, <sup>5</sup> gm.	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Liver extract *	(4)	(4)	(4)	(4)	(4)	(4)										
Thiamine, mg.	.3	.3	.3	.3	.3	.3										
Pyridoxine, mg.	.4	.4	.4	.4	.4	.4										
Pantothenic acid, mg.	.5	.5	.5	.5	.5	.5										
Nicotinic acid, mg.	5	5	5	5	5	5										
Choline, mg.	100	100	100	100	100	100										

All rats fed the above rations received 2 drops of haliver oil per week.

<sup>1</sup> Extracted continuously for 3 days with hot 95% ethanol.<sup>2</sup> Cell-U flour.<sup>3</sup> Washed 3 times with hot water.<sup>4</sup> Borden's purified casein, supplied under the trade name "Labco".<sup>5</sup> Hegsted et al. ('41).

\* Riboflavin-free liver extract (Wagner et al., '40). Mixed into the ration in amounts equivalent to 4% of the original liver powder.

\* Rats receiving these rations were given a daily oral supplement of the following vitamins (μg.) dissolved in 1 ml. of a 25% ethanol solution: thiamine, 40; pyridoxine, 50; pantothenic acid, 150; nicotinic acid, 500; and choline, 10,000.

ferences could be shown between the groups receiving lard and Crisco. If the gains in weight of the rats fed each of the rations are plotted against the daily riboflavin dosage (omitted here to conserve space), it can be shown that increasing the fat in the diet to 40% at the expense of dextrin had an effect of increasing the riboflavin requirement about 4 to 6  $\mu\text{g.}$  per day. Similarly, the feeding of rations containing 25% fat increased the requirement by about 2  $\mu\text{g.}$  per day. The economy of food utilization, calculated as grams gained per 100 Cal. consumed, is closely correlated with the responses in growth made by the rats receiving the different rations and varying amounts of riboflavin.

TABLE 2

*Growth of rats receiving rations containing different percentages of fat and graded amounts of riboflavin. (Each figure represents the average response of 3 rats.)*

RATION NO.	TYPE OF RATION	DAILY RIBOFLAVIN INTAKE IN MICROGRAMS	GRAMS GAINED IN 7 WEEKS <sup>1</sup>	GRAMS GAINED PER CALORIE FED $\times 100$ (7 WEEKS)
K-24	High dextrin	0	12	1.1
		3	72	5.7
		6	102	8.9
		9	148	7.1
		12	170	8.0
	Average		101	5.8
K-25	25% lard	0	10	.7
		3	41	4.0
		6	71	5.4
		9	88	6.6
		12	121	7.1
	Average		66	4.8
K-26	25% Crisco	0	8	.9
		3	39	4.1
		6	61	5.7
		9	98	6.6
		12	116	6.6
	Average		65	4.8
K-27	40% lard	0	4	.4
		3	16	2.1
		6	53	4.8
		9	84	5.9
		12	98	6.9
	Average		51	4.1

<sup>1</sup> Total grams gained during 7 weeks following 2-week depletion period on riboflavin low ration K-24.

That a 40% level of fat in the diet does not restrict the growth of rats when adequate riboflavin is supplied was demonstrated by raising two groups of four weanling male rats on rations K-24 and K-27 plus 100  $\mu$ g. of riboflavin per day. At the end of 6 weeks the animals fed the high dextrin ration showed an average weight increase of 184 gm. while those receiving the high fat diet had gained an average of 189 gm.

It was conceivable that in riboflavin deficient rats the deleterious effect of fat might be due to an impairment of fat absorption. In order to test this possibility fat balance studies were conducted during the third and fourth weeks following the depletion period on rats receiving the high dextrin (K-24) and high fat (K-27) rations. Fat retention was excellent in all animals regardless of the type of ration fed or of the riboflavin intake. The average retention of the fat of ration K-24 was 96% (95 to 99) and of ration K-27, 98% (97 to 99).

The total fat content of the livers of all the rats was determined in an attempt to detect a disturbance in fat metabolism or transport. Liver fat on a dry basis varied between 1.6 and 5.9%. The variations could not be associated with either the amount of riboflavin or the type of ration fed.

#### *Effect of substituting fat for sucrose*

As mentioned previously, the effect of dietary fat in increasing the riboflavin requirement might be due not to the fat itself but to the replacement of dextrin, a carbohydrate known to stimulate the synthesis of vitamins by intestinal bacteria. Sucrose apparently lacks a similar stimulatory property. If the problem is essentially one of bacterial synthesis, the use of sucrose rather than dextrin as the source of carbohydrates should reduce growth, and the substitution of fat for sucrose should not result in a further reduction. With this in mind the following experiment was designed.

Groups of sixteen male weanling rats were partially depleted of their riboflavin reserves by feeding for 2 weeks the following riboflavin-low rations (table 1): K-24 (high dextrin), K-27 (40% fat, dextrin), K-28 (high sucrose), and K-29 (40% fat, sucrose). The animals within each group were then given varying amounts of riboflavin, namely, 0, 6, 12, 24, and 36  $\mu$ g. per day. The growth responses of the rats during the 6-week experimental period including the first 2 weeks of depletion are given in table 3.

The superiority in growth of rats receiving the high dextrin ration over animals fed the dextrin-lard ration is again substantiated. This same relationship cannot be shown when sucrose is employed as the



source of carbohydrate. This is in agreement with the observations of Potter, Axelrod and Elvehjem ('42) who were unable to show any effect of fat on the riboflavin requirement of dogs fed sucrose rations. The growth of rats fed the high sucrose ration was not significantly greater than that of animals fed either of the high fat diets. Rats fed the lard-dextrin ration, K-27, grew no better than those receiving the sucrose or lard-sucrose diets although this diet contains considerable amounts of dextrin. It would appear that fat reduces or eliminates the riboflavin sparing effect of dextrin when these two constituents are included in the same ration.

TABLE 3

*Growth of rats receiving different riboflavin-low diets and various levels of riboflavin.  
(Total grams gained in 6 weeks, including 2-week depletion period.)*

RATION NO.	TYPE OF RATION	MICROGRAMS OF RIBOFLAVIN/DAY <sup>1</sup>				
		0 (4)	6 (3)	12 (3)	24 (3)	36 (3)
K-24	Dextrin	33	69	88	159	159
K-27	Lard-dextrin	4	40	75	116	124
K-28	Sucrose	21	43	89	121	149
K-29	Lard-sucrose	12	46	75	128	153

<sup>1</sup> Number of rats indicated by numbers in parentheses.

The comparison of the sucrose and high-fat sucrose rations was repeated by raising two groups of five young male rats on rations K-28 and K-29 plus a suboptimal daily dose of 6 µg. of riboflavin. The preliminary depletion period was omitted. Food consumption records were kept throughout the 7-week experimental period. The rats fed the high-sucrose ration gained an average of 82 gm. and those receiving the 40% fat-sucrose diet showed a weight increase of 76 gm. The utilization of the two rations was comparable, 5.9 and 5.4 gm. gained per 100 Cal. consumed of rations K-28 and K-29, respectively.

*Effect of various carbohydrates and of fat on the requirement  
for and fecal excretion of riboflavin*

The above work suggests that the beneficial effect of dextrin is due to its stimulation of the production of available riboflavin by the intestinal flora of the rat. If this concept is correct, a positive correlation might be found between growth and the amount of riboflavin excreted in the feces or contained in the cecum. The size of the cecum might also be related to the amount of riboflavin synthesized. The following experiment was designed to determine whether or not such correlations could be made.

A total of 78 three-week-old male rats were fed isocalorically equivalent rations differing only in the kind of carbohydrate or in the amount of lard present. The following types of diets were employed (table 1): high-sucrose (K-30), 40% fat, sucrose (K-31), high dextrin (K-32), 40% fat, dextrin (K-33), 40% lactose, sucrose (K-34), 40% fat, lactose (K-35), high-sucrose to which 10 parts of cellulose per 100 parts of ration had been added (K-36), 40% fat, sucrose, cellulose (K-37), high commercial cornstarch from which the dextrin had been prepared (K-38), and high alcohol-extracted dextrin (K-39). The last ration was fed to determine whether or not alcohol extraction would remove any hypothetical substance responsible for the stimulatory effect of dextrin. All animals received 6  $\mu$ g. of riboflavin per day throughout the 6-week test period in order to insure consistent yet suboptimal growth. Half of the rats received the fullers' earth-treated butanol liver extract equivalent to 4% of the original liver powder, but because these animals responded no differently than did non-supplemented animals of the same group, no differentiation is made between the rats within each group. The rations containing high levels of fat were prepared daily and fed in clean food dishes to prevent rancidity.

At the end of 6 weeks the feeding of riboflavin was discontinued. The rats were maintained for an additional 9 days and throughout the last week of this period the feces were collected daily and stored in the refrigerator. The animals were then killed and the cecal contents collected quantitatively. The feces and cecal contents were dried *in vacuo* at 55°C., weighed, and saved for later riboflavin analysis.

The riboflavin content of the feces of individual rats was determined in duplicate using essentially a combination of the fluorometric methods of Hodson and Norris ('39) and Conner and Straub ('41) as outlined by Andrews ('43). Because of the small quantities of material available, the cecal contents of the animals of each group were combined for analysis. Several samples of feces were assayed by the bacteriological method of Snell and Strong ('39) as well as by the fluorometric method. The values obtained by the former procedure were erratic and as much as two or three times higher than those obtained by fluorometric analysis. This discrepancy was eliminated, however, by employing the Strong and Carpenter ('42) modification of the bacteriological assay which involves the removal of fatty stimulatory substances by means of ether extraction. The average riboflavin content of twenty samples of feces as measured by the fluorometric and bacteriological procedures was, respectively, 27.6 and 26.3  $\mu$ g. per gram.

Lamoreux and Schumacher ('40) have reported that the riboflavin content of chicken excreta increases as much as 100% in 24 hours on standing at room temperature. That a similar synthesis of riboflavin did not occur in the rat feces during the collection intervals was shown indirectly by comparing the riboflavin content of the formed fecal material from the lower colon, obtained when the animals were autopsied, with that of the feces that had been collected throughout the preceding week. The colonic "feces" of eight animals taken at random from the various groups contained an average of 30.1  $\mu\text{g.}$  per gram while the dropped feces from the same rats had a comparable riboflavin content of 32.9  $\mu\text{g.}$  per gram. Active riboflavin synthesis probably does not occur in rat feces on standing because of their relatively low moisture content.

TABLE 4

*Effect of various carbohydrates and of fat on growth and on the fecal excretion of riboflavin. All rats received 6  $\mu\text{g.}$  of riboflavin per day except during period of feces collection.*

RATION NO.	TYPE OF RATION	NO. OF RATS	GAIN IN 6 WKS.	RIBO-FLAVIN/GM. OF FECES	RIBO-FLAVIN EX-CRETED IN FECES /WK.	RIBO-FLAVIN EX-CRETED IN FECES /WK./ 100 GM. RAT	RIBO-FLAVIN/GM. OF CECAL CON-TENTS	CECAL CON-TENTS/ 100 GM. OF RAT
			gm.	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	$\mu\text{g.}$	gm.
K-30	Sucrose	14	80	25.0	23.2	19.3	22.0	.15
K-31	Lard-sucrose	8	75	23.5	24.5	21.7	19.0	.16
K-32	Dextrin	8	108	34.3	72.4	50.3	25.6	.25
K-33	Lard-dextrin	8	68	24.3	24.3	22.8	19.5	.20
K-34	Lactose-sucrose	6	89	45.3	89.0	67.5	41.8	.49
K-35	Lard-lactose	6	87	23.4	50.6	38.8	25.6	.35
K-36	Cellulose-sucrose	6	83	6.7	28.3	23.1	9.7	.26
K-37	Lard-cellulose-sucrose	6	81	6.4	30.2	24.9	8.2	.24
K-38	Cornstarch	8	98	30.5	43.9	32.5	21.7	.14
K-39	Alcohol extracted dextrin	8	97	29.5	58.4	42.3	24.7	.31

It was obviously important to determine whether or not significant quantities of riboflavin were being contributed by the variable constituents of the diets. Lard, sucrose, cornstarch and lactose were found to contain less than 0.1  $\mu\text{g.}$  of riboflavin per gram as measured by the fluorometric method, amounts that could not seriously affect the results of this experiment. The results are given in table 4.

The growth of rats receiving the high dextrin, cornstarch, and alcohol-extracted dextrin diets was superior to that of animals fed any of the other rations. Again the substitution of lard for a large part of the dextrin of the diet had a deleterious effect on growth while a similar

replacement of sucrose by lard was without effect. The inclusion of cellulose in the sucrose and lard-sucrose rations did not improve growth appreciably. The replacement of a large part of the sucrose of ration K-30 with lactose resulted in a slight increase in growth.

With the exception of the lactose-fed groups, growth is roughly proportional to the amount of riboflavin excreted per week in the feces. Similarly, but to a less marked degree, those groups of rats which exhibited the best growth, produced feces containing the greatest amount of riboflavin per gram. About the same amount of fecal riboflavin was excreted weekly by the animals receiving the high sucrose, fat-sucrose, fat-dextrin, and the cellulose diets. Although the riboflavin content of the feces of animals fed the cellulose diets was very low, the large amount of feces voided made the total excretion comparable to that of rats fed the high-sucrose diet without cellulose.

The feeding of the lactose-sucrose diet resulted in the greatest fecal production of riboflavin. According to the above correlation made between growth and the amount of riboflavin found in the feces, one might have expected the growth of the lactose-fed rats to have been correspondingly above that of the other animals, but this was not the case. It is possible, however, that when lactose was fed, the suboptimal riboflavin intake was not the primary growth-limiting factor. The data of Boutwell, Geyer, Elvehjem and Hart ('43) show that on a diet containing 48% lactose and an abundance of all the known vitamins, rats do not grow as well as when other carbohydrates are employed. When lard was substituted for the sucrose and part of the lactose of lactose-sucrose ration, K-34, the total fecal riboflavin was greatly reduced. Likewise the replacement of a substantial part of the dextrin of ration K-32 with lard resulted in a decreased fecal riboflavin output. The relatively low degree of intestinal synthesis of riboflavin which results when the high-sucrose (K-30) and sucrose-cellulose (K-36) rations are fed was not further decreased by the inclusion of high levels of fat in these diets.

With certain exceptions, the total amount of riboflavin excreted in the feces is related to the size of the cecum as measured by the dry weight of the cecal contents. The ceca of lactose-fed rats were greatly distended and contained whitish material, presumably lactose. The feeding of dextrin rations K-30 and K-39 also resulted in large ceca. It was in these animals that the greatest synthesis of riboflavin occurred. On the other hand, the feeding of cornstarch was responsible for abundant fecal production of riboflavin even though large ceca were not found. Conversely, the intestinal synthesis of riboflavin in

cellulose-fed rats was not great although the cecal size per 100 gm. of these animals was about the same as that of the rats receiving the high-dextrin ration. Apparently the amount of riboflavin excreted in the feces is as dependent upon the type as upon the quantity of material in the cecum. Although somewhat lower in most instances, the riboflavin values of the cecal contents are a reflection of those of the feces.

*Effect of a high dietary intake of fat on the deficiency symptoms and survival of riboflavin deficient rats*

It has been shown previously (Mannering et al., '41) that rats receiving a high fat-dextrin diet deficient in riboflavin develop a spastic paralysis of the hind quarters, a condition that does not occur with any marked severity when a high dextrin ration is fed. This same relationship of paralysis to fat intake can be shown when sucrose rather than dextrin is employed as the source of carbohydrate.

One group of rats weighing between 65 and 75 gm. received the basal high-sucrose ration K-30 and another, the lard-sucrose ration K-31. Animals of this size were employed in order to produce a chronic rather than the more acute state of deficiency which results when smaller rats are used, a higher incidence of spastic paralysis resulting when the deficiency is prolonged over many weeks. Animals were allowed to continue on experiment for 133 days throughout which time no riboflavin was administered. The high fat ration was prepared and fed daily to prevent rancidity.

Of six rats fed the high-sucrose diet three were still alive after 133 days. The average survival time was 118 days (83-133 days). Although several of these rats showed minor symptoms of spasticity before dying, in no instance could the condition be considered severe. None of the seven rats fed the lard-sucrose diet survived the 133-day period and the average survival time was 81 days (50-126 days). All of these rats developed severe paralysis.

These results were confirmed in a similar experiment. Eight of twelve rats fed the high carbohydrate diet survived the 133-day period, the average survival time being 113 days (85-133 days), as compared to 91 days (66-125 days) for twelve animals receiving the high fat diet. Again severe spasticity occurred only in rats fed the high lard ration. Three of the rats of each group were given 2  $\mu$ g. of biotin per day (biotin liver concentrate) and three others received 10 mg. of inositol per day. Neither of these substances had any influence on survival or the deficiency symptoms. That the extremely emaciated condition of the

deficient rats was not responsible for the paralysis was shown by maintaining the weights of six rats receiving the high fat diet plus 100  $\mu$ g. of riboflavin per day equal to the weights of six of the above rats fed the same ration without riboflavin by limiting the food intake of the former. Although emaciated; the starved animals receiving abundant riboflavin remained outwardly in excellent condition.

In addition to aggravating the production of spastic paralysis and decreasing the survival time the feeding of a high fat diet results in a more severe dermatitis and alopecia than is obtained when a high sucrose diet is fed. Denuded and incrustated areas are particularly noted about the face and limbs. Animals in the advanced stages of the deficiency have been cured by feeding 100  $\mu$ g. of riboflavin per day. By the end of 2 or 3 weeks the spasticity completely disappears. Large clumps of a mixture of hair, skin and ration are pulled off by the rat leaving practically the entire surface of the animal bare. Gradually the hair grows in and the rat appears normal in all respects except that a few scars remain, especially about the face where incrustation is usually severe.

A condition known as "spectacle eye", characterized by a local alopecia immediately surrounding the eyelids of rats, has been related to biotin (Nielson and Elvehjem, '41) and to inositol (Paveek and Baum, '41) deficiency. Chiek, Maerae and Worden ('40) have noted a similar loss of hair in riboflavin deficient rats. A majority of the animals in our experiments developed this peculiar hair loss about the eyes which could not be prevented by the daily administration of 2  $\mu$ g. of biotin or 10 mg. of inositol. These rats were commonly observed in the act of rubbing their eyes which probably accounts for the observed "spectacle eye".

The reddish accumulation of porphyrin on the paws, nose and whiskers of riboflavin deficient rats described by Chiek et al. ('40) was observed in all but a small percentage of the above animals. The number of rats showing cataract was two out of eighteen fed the high carbohydrate diet and six out of nineteen receiving the high fat ration.

#### DISCUSSION

It is apparent from the foregoing experiments that dextrin and cornstarch reduce the dietary riboflavin requirement of the rat and that sucrose, cellulose, or fat do not share this property. Dextrin and cornstarch probably increase the amount of available riboflavin synthesized in the intestine by providing the intestinal microorganisms with a favorable medium for such synthesis, by increasing the number of

microorganisms, or by changing the flora to a type capable of producing greater quantities of the vitamin. The incomplete digestion of dextrin or cornstarch allows some of the carbohydrate to reach the lower regions of the tract where microorganisms are ordinarily found in abundance. Sucrose is more readily digested and absorbed than dextrin or cornstarch and would not be expected to reach this site of increased bacterial activity in any great quantity. This concept is in keeping with that suggested by Guerrant et al. ('35). Fat and cellulose, though capable of reaching the lower tract, are apparently of little value in promoting the synthesis of available riboflavin. In fact, fat may inhibit such synthesis.

The position of lactose in sparing riboflavin is uncertain, allowing growth intermediate between that obtained on the sucrose and dextrin diets. Morgan, Cook and Davison ('38) have reported that the feeding of lactose reduces the riboflavin requirement below that of animals fed either sucrose or cornstarch and were able to show that the ceca of lactose-fed rats contained appreciable amounts of riboflavin. Contrary to our observations, their data do not indicate that cornstarch supports better growth than does sucrose when the riboflavin intake is limiting.

Numerous investigators in the past have noted that the feeding of vitamin-low rations containing certain constituents results in greater growth than can be accounted for by the vitamin content of these constituents, and they have attempted to correlate such unusual growth with an increased vitamin content of the feces or of the material found in various sections of the digestive tract. Most of this work has not been of a strictly quantitative nature due largely to inadequacy of vitamin determination methods and to the inability to properly differentiate between the individual components of the B complex.

The idea has prevailed that a measurement of the vitamins in the tract and in the feces could somehow be used as a relative measure of the amount of the vitamin made available to the animal through bacterial synthesis. That such a relationship must necessarily exist is untenable on theoretical grounds. It can be successfully argued that the vitamin content of the feces or of other intestinal material may have little to do with the amount of the vitamin absorbed by the host. For example, the riboflavin may be synthesized in a region of the intestine from which it cannot be absorbed. Selye ('43) has studied the absorption of riboflavin from the various sections of the tract, separated by ligatures, using nephrectomized rats. He concludes that in the small intestine, riboflavin is both absorbed and excreted, while in the cecum and colon, injected riboflavin is rapidly destroyed with little,

if any, absorption of the vitamin. However, in his experiments very large concentrations of riboflavin were employed, and only amounts of the vitamin that could be detected grossly were considered. Whether or not minute amounts of riboflavin, such as are involved under normal conditions, can be absorbed from the cecum and colon still remains a question. That the cecum does actually contribute to the supply of the rat's B vitamins has been demonstrated in the cecectomy studies of Taylor, Pennington and Thacker ('42). The cecum has been emphasized as the most probable site of bacterial synthesis largely because of the abundance of microorganisms found there. However, other sections of the tract cannot be ignored in this respect. Porter and Rettger ('40) find, contrary to much prevailing opinion, that the upper intestine of the rat contains appreciable numbers of viable bacteria.

Secondly, the vitamin may exist as an integral part of the bacteria, unavailable to the animal. Abdel-Salaam and Leong ('38) have demonstrated that the thiamine synthesized by the mixed flora taken from the ceca of rats and grown in vitro is contained in the bacterial cells rather than in the surrounding medium, and Mitchell and Isbell ('42) find that the greater proportion of the vitamins found in the cecal contents of rats is contained in the bacterial cells. However, it has been shown that when bacteria, capable of synthesizing vitamins, including those species normally found in the tract, are grown in pure cultures, the vitamins synthesized are found largely in the medium (Burkholder and McVeigh, '42, Thompson, '42, Rodgers, '42). The vitamins elaborated are a result of both bacterial excretion and autolysis. This difference between the amount of free vitamin obtainable when mixed and when pure cultures were used is understandable if, in the mixed cultures, bacteria requiring or destroying an external source of the vitamin are present as well as those capable of its synthesis, in which case, the former would utilize the vitamin released by the latter. Comparable symbiotic relationships may be expected to exist in the cecum and other parts of the tract containing a mixed flora.

The contents of the tract may be considered to be in constant state of activity, bacteria constantly dividing, dying, disintegrating, and giving up their contents for digestion and absorption. Organisms may be excreting vitamins directly into the intestinal media to be absorbed with varying degrees of rapidity by either the host or by still other bacteria requiring the vitamin. The state of the intestinal contents at any given instant, then, will be the result of the relative rates at which the many possible processes proceed. A small quantity of either total or available vitamin in the intestinal material or feces might be inter-



microorganisms, or by changing the flora to a type capable of producing greater quantities of the vitamin. The incomplete digestion of dextrin or cornstarch allows some of the carbohydrate to reach the lower regions of the tract where microorganisms are ordinarily found in abundance. Sucrose is more readily digested and absorbed than dextrin or cornstarch and would not be expected to reach this site of increased bacterial activity in any great quantity. This concept is in keeping with that suggested by Guerrant et al. ('35). Fat and cellulose, though capable of reaching the lower tract, are apparently of little value in promoting the synthesis of available riboflavin. In fact, fat may inhibit such synthesis.

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Numerous investigators in the past have noted that the feeding of vitamin-low rations containing certain constituents results in greater growth than can be accounted for by the vitamin content of these constituents, and they have attempted to correlate such unusual growth with an increased vitamin content of the feces or of the material found in various sections of the digestive tract. Most of this work has not been of a strictly quantitative nature due largely to inadequacy of vitamin determination methods and to the inability to properly differentiate between the individual components of the B complex.

The idea has prevailed that a measurement of the vitamins in the tract and in the feces could somehow be used as a relative measure of the amount of the vitamin made available to the animal through bacterial synthesis. That such a relationship must necessarily exist is untenable on theoretical grounds. It can be successfully argued that the vitamin content of the feces or of other intestinal material may have little to do with the amount of the vitamin absorbed by the host. For example, the riboflavin may be synthesized in a region of the intestine from which it cannot be absorbed. Selye ('43) has studied the absorption of riboflavin from the various sections of the tract, separated by ligatures, using nephrectomized rats. He concludes that in the small intestine, riboflavin is both absorbed and excreted, while in the cecum and colon, injected riboflavin is rapidly destroyed with little,

hydrates which decrease the amount of dietary riboflavin needed by the rat for growth are also responsible for the greatest quantities of riboflavin in the feces. However, the fecal excretion of riboflavin was greatest when lactose was fed, although the growth-promoting effect of lactose was not great.

The relationship between growth, cecal size, intestinal synthesis, and the quantity of riboflavin in the cecum and feces is discussed.

The feeding of high levels of dietary fat to riboflavin deficient rats results in a spastic paralysis of the hind quarters, a condition not noted with any degree of severity when riboflavin-low rations of high carbohydrate content are employed. Riboflavin deficient rats survive for shorter periods of time when fed a high fat ration than when maintained on a high carbohydrate diet.

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# FOOD UTILIZATION AND APPETITE IN RIBOFLAVIN DEFICIENCY<sup>1</sup>

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## THREE FIGURES

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Several papers have appeared recently dealing with riboflavin as a factor affecting the efficiency of food utilization in the rat. Sure and Dichek ('41) and Sure ('41), employing a paired-feeding technique, have shown that riboflavin has a pronounced effect on the utilization of food for the synthesis of body tissues. It was further demonstrated that, unlike in a thiamine deficiency, the final collapse of the riboflavin deficient rat is not associated with any great decrease in food intake. Voris, Black, Swift and French ('42), using moderately deficient animals, have made similar observations.

In the paired-feeding studies of the above-mentioned authors, animals receiving an adequate diet were limited in their food intakes to the amounts eaten by animals fed the deficient diet. Here the efficiency of food utilization was measured by a comparison of the growth of the two groups of animals. It is also possible to obtain information regarding food utilization by means of ad libitum feeding if groups of animals are given graded, suboptimal levels of the vitamin in question and the amount of growth per calorie of food consumed computed for each group. During the course of the experiments described in the preceding paper (Mannering, Orsini and Elvehjem, '44) we were able to study the effect of riboflavin on food utilization using this latter procedure.

The composition of the riboflavin-low rations has already been given (Mannering et al., '44). Three-week-old male albino rats weighing 35 to 40 gm. were partially depleted of their riboflavin reserves for 2

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weeks on ration K-24 before being divided into four groups of twelve animals receiving rations K-24, K-25, K-26, and K-27 and varying amounts of riboflavin (0, 3, 6, 9, and 12  $\mu$ g. per day). These rations differ only in the percentage or type of fat present and are calorically equivalent. Food consumption records were kept throughout the 7-week supplementation period. In figure 1 both the average grams gained per day and the average grams gained per 100 Cal. fed have been plotted against the number of micrograms of riboflavin fed per day. To conserve space the data for all rats receiving a given level of riboflavin were averaged together regardless of the type of ration fed. This was justifiable since equal numbers of rats were employed at the various riboflavin levels for each of the rations. The curves shown are qualitatively similar to those obtained when the four groups are considered separately.

It is seen that as the daily administration of riboflavin is increased, less food is necessary for a given increment of growth. The curve representing growth is linear within the limits of 0 and 12  $\mu$ g. of riboflavin per day and would be expected to level off if greater amounts of the vitamin were fed. The configurations of the curves indicate that food utilization approaches a maximum at a lower level of riboflavin intake than does growth. When the average grams gained per 100 Cal. fed was plotted against the logarithm of the number of micrograms of riboflavin fed per day, a straight line was obtained (fig. 1).

The marked effect of riboflavin in supporting the utilization of food for growth cannot be accounted for by a lack of absorption. The feces of all rats receiving rations K-24 and K-27 were collected during the third and fourth weeks following the depletion period, dried, and weighed. The total digestibility of these rations was excellent. The average grams of feces obtained per 100 gm. of ration K-24 fed was 3.1 (1.2-4.8) and of ration K-27, 5.2 (4.0-6.4). Variations bore no relation to the riboflavin intake.

A second experiment was devised for the purpose of studying the effect of riboflavin on the efficiency of food utilization for maintenance. A method of "paired-weighing" was employed whereby an animal receiving a complete diet was restricted in food intake so that its weight remained equal to that of an ad libitum-fed rat receiving a diet deficient in riboflavin. A comparison of the efficiency of food utilization was then based on the amount of food necessary to maintain each of the animals at the same weight. So little growth occurs in a rat completely deprived of riboflavin that food is used almost entirely for maintenance.

Six male albino rats weighing between 65 and 75 gm. were fed 100  $\mu$ g. of riboflavin per day and just enough of ration K-31 to keep their

weights equal to the weights of six similar rats fed the same ration *ad libitum* but receiving no riboflavin. By means of daily weighings and careful allocation of food it was possible to keep pairs of starved and deficient rats within 3 gm. of each other. During the eleventh week three of the deficient animals died while the remaining three collapsed during the fifteenth, sixteenth and seventeenth weeks. All of the starved animals remained in apparent good condition throughout the experiment

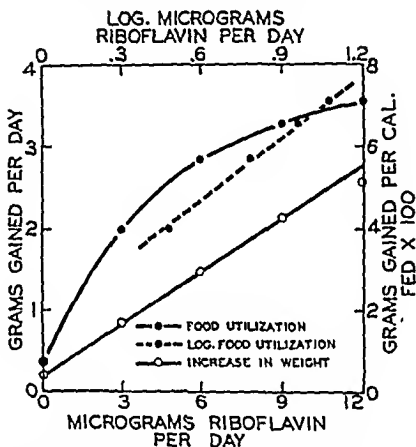


Fig. 1 Curves showing effect of progressively higher levels of riboflavin on growth and food utilization of rats during a 7-week period following 2 weeks of depletion on a riboflavin-low diet. Each point represents twelve rats.

despite extreme emaciation. In figure 2 are shown the growth curves and weekly food intakes of both deficient and starved rats for the first 10 weeks of the experiment.

After the first week the starved animals required progressively less food to maintain their weights equal to those of the riboflavin deficient rats until by the tenth week the latter were consuming an average of 1.8 (1.2-2.8) times more of the ration than the former. During the entire experimental period of 17 weeks the avitaminotic rats ingested an average of 1.5 (1.3-1.7) times as much food as the starved controls. The food utilization during the fifth through the eighth weeks is more strictly representative of food utilization for maintenance than during any other period of the experiment because the animals were neither

gaining nor losing weight at this time. Throughout this growth plateau period the rats that were deprived of riboflavin consumed an average of 1.6 (1.3-1.9) times the amount of food eaten by their control mates.

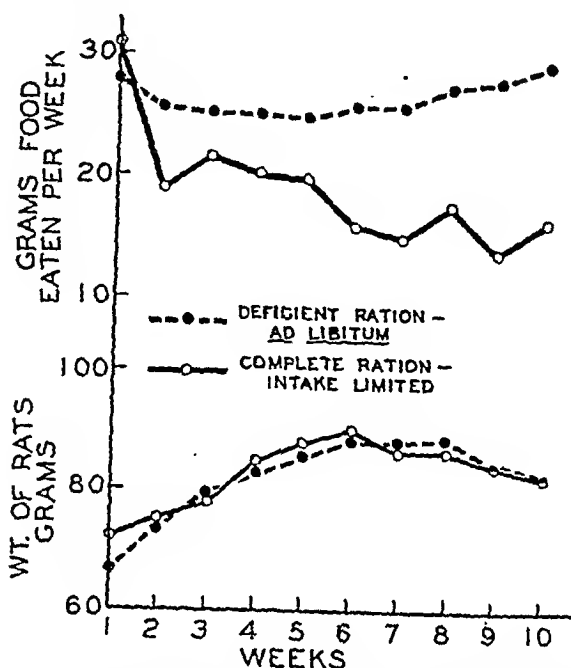


Fig. 2 Curves showing growth and food consumption of riboflavin deficient rats and of rats receiving a limited intake of an adequate diet. Each curve represents six rats.

Figure 3 shows the weekly food consumption per 100 gm. of rat of both starved and deficient animals. During the first 4 weeks of the experiment the rats deprived of riboflavin consumed progressively less food per 100 gm. of weight and then progressively more as the deficiency became more severe. The period of increased efficiency of food utilization occurred while the animals were slowly growing, while the later decrease in efficiency corresponded to the period when the rats were losing weight. The starved animals became continuously more efficient in their utilization of food as the period of restricted food intake was prolonged.

Rats that are limited in food intake but supplied with sufficient riboflavin were observed to be much more active than deficient rats. Unless the energy output of the animal is known, it is, of course, impossible to obtain absolute values for the influence of riboflavin on food utilization for maintenance. Because the starved animals were apparently more active than the vitamin deficient rats, the effect of

riboflavin in promoting the utilization of food is probably even greater than has been indicated.

It has been previously mentioned that certain workers have been unable to find any appreciable inanition in riboflavin deficiency. The following study indicates that moderate inanition does occur in most animals suffering from a lack of riboflavin.

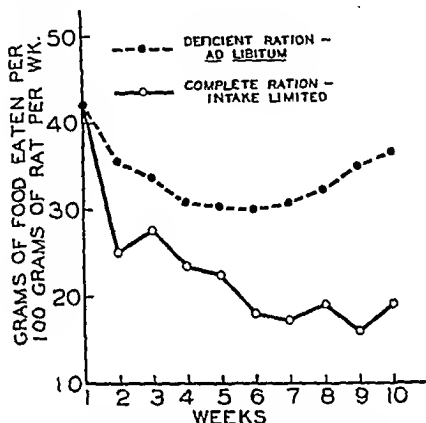


Fig. 3 Curves showing the grams of food consumed per 100 gm. of rat per week of starved and of riboflavin deficient rats. Each curve represents six rats.

Male albino rats weighing about 65 gm. were divided into two groups, one of which received ration K-30, and the other, ration K-31. No riboflavin was fed during the 19-week experimental period. The series was repeated, but as a separate consideration of either the two series or of the animals receiving the different rations did not add to the information gained, the results have been combined. A total of thirty-seven rats were employed in this experiment. By the end of the fifth week of depletion the rats were consuming an average of 20% less calories than they were during the first week of the experiment. On a calculated basis of Cal. eaten per 100 gm. of rat, an average decrease of 35% was observed during the fifth week. The selection of the fifth week for purposes of comparison was arbitrary and does not necessarily represent the period of lowest food intake. During the nineteenth week, eleven surviving animals were consuming an average of 27% less calories than they were during the first week, an average decrease of 31% per



100 gm. of rat. Considerable variation was noted in the degree of anorexia, some rats even showing an increased appetite. An example of this variation is evident in figure 2, where, by coincidence, the six rats used in the experiment represented by the graph did not show appreciable inanition.

It was often observed that rats markedly increased their food intake for a period of a few days prior to dying of the deficiency. This has also been reported by Sure ('41). The increase in food consumption during the last 7 days before death over that of the preceding 7 days was calculated and found to average 11%. The average, however, does not well represent those animals which showed increases just previous to dying in that several of the rats actually decreased their food intake. Increases as high as 62% were observed.

#### DISCUSSION

The presented data clearly demonstrate that riboflavin has a profound effect on food utilization both for growth and for maintenance. It has been further shown that a lack of riboflavin is not associated with severe anorexia, the animals consuming considerable quantities of food even in the later stages of the deficiency. The question arises as to the fate of the ingested food, a large proportion of which, although absorbed, cannot be accounted for as being utilized for growth or for maintenance. The most logical explanation for the poor food economy in riboflavin deficiency is that the intermediate products of metabolism are probably wasted through incomplete combustion. The role of riboflavin in the function of enzyme systems controlling cellular respiration has been well established (Elvehjem and Wilson, '40; Potter, '40; and Green, '41). This view also gains support from the observation of Orsini, Waisman, and Elvehjem ('42) that the respiratory quotient of the riboflavin deficient rat is abnormally high.

#### SUMMARY

Riboflavin plays an important role in the economy of food utilization both for growth and for maintenance.

Moderate inanition occurs in riboflavin deficiency but is not responsible for the death of the deficient rat since a relatively high level of food consumption is maintained throughout the entire period of avitaminosis. The food intake often increases during the last few days preceding death.

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# ADEQUACY OF SIMPLIFIED DIETS FOR THE PIG

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## ONE FIGURE

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The literature on the nutritional requirements of swine is too voluminous for a detailed review but it has been shown that these animals require vitamins A (Hughes, Aubel, and Lienhardt, '28) and D (Johnson and Palmer, '41), thiamine (Wintrobe, Mitchell, and Kolb, '38), riboflavin (Hughes, '40), pyridoxine (Hughes and Squibb, '42), pantothenic acid (Hughes and Ittner, '42), and nicotinic acid (Chick, Macrae, Martin and Martin, '38 a). Hughes and collaborators ('42) reported normal growth in swine that did not receive choline and according to Wintrobe and coworkers ('42) it is uncertain that swine require this substance.

The first use of simplified diets for studies on the nutritional requirements of swine, was published by Chick, Macrae, Martin, and Martin ('38 b). Vitamins A and D were supplied in cod liver oil. Thiamine, riboflavin, and nicotinic acid were supplied as crystalline compounds. The pigs were 12 weeks of age when the experimental period began and they weighed from 51 to 70 lb. Gains in weight were normal for a short time but ceased abruptly after 3 or 4 weeks. If 4% of dried yeast was incorporated in the diet growth was continuous.

Wintrobe ('39) also was successful in rearing pigs on synthetic diets, though the water-soluble vitamins were supplied in dried yeast. It is noteworthy that he started the pigs at an earlier age than did the investigators previously mentioned. The pigs were from 2 to 23 days of age in the beginning, and they were retained until they were approximately 8 months old. During the first 3 months they gained in weight very slowly, but following that time they began to grow normally. Wintrobe, Miller, Follis, Stein, Mushatt, and Humphreys ('42) repeated this observation, with minor changes in procedure. The yeast feeding was discontinued when the pigs were approximately 12 to 15 weeks of age, and synthetic vitamins were included in the diet. These

<sup>1</sup> Contribution from the Missouri Agricultural Experiment Station, Journal Series no. 953.

included thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, and choline. The pigs grew fairly well for about 6 months, though far under the normal rate. One would conclude that the diet was not entirely adequate.

Hughes and coworkers ('42) used a basal diet composed of casein 15, sucrose 81, and salts 4, but their animals grew normally although they received no crude vitamin carriers. Vitamins A and D were supplied in cod liver oil. The water soluble vitamins included were thiamine, riboflavin, pyridoxine, pantothenic acid, and nicotinic acid. It should be noted, however, that the animals weighed about 30 lb. when the feeding test began and presumably this was shortly after they had been weaned.

#### EXPERIMENTAL

The mortality had been excessively high during preliminary trials, in which the new-born pigs were removed from the sows at birth, and it was assumed that the failure to receive colostrum was at least partly responsible for the deaths. The pigs were therefore allowed to

TABLE 1

*Illustrative basal ration — ration 308 — used for feeding experiments with pigs.*

INGREDIENT		VITAMIN <sup>1</sup> SUPPLEMENTS	
	%		mg./100 gm. diet
Acid washed casein	30	Thiamine	0.8
Sucrose	30	Riboflavin	1.6
Corn starch	5	Pyridoxine	1.2
Lard	30	Ca-pantothenate	2.0
Salt mixture <sup>2</sup>	5	Percomorph oil	50.0 (3000 I. U. vitamin A, 850 I. U. vitamin D)
		Nicotinic acid	2-4
		Alpha tocopherol	4
		K <sub>2</sub>	2
		Choline	200-300

<sup>1</sup> Generously supplied by Merck and Co., Rahway, New Jersey.

<sup>2</sup> 99.2% of the salt mixture 351 of Hubbell, Wakeman and Mendal ('37), 0.75% manganous sulfate, 0.05% of zinc sulfate.

nurse their dams for the first 2 days after birth and were then given the experimental diets. These were all of the simplified type. The diet supplied to most of the pigs contained the four fat-soluble vitamins and six members of the vitamin B complex. A typical example is described in table 1.

Diet 308 was modified at various times but apparently one diet was as useful as another. The salt mixture of Hubbell, Wakeman, and

Mendel is low in phosphorus, but the addition of more phosphorus did not improve the diet. Some attention to detail is necessary to convert the diet into a liquid that is suitable for very young animals. The casein was added to warm water, with enough sodium hydroxide (0.75 gm. of the alkali per 30 gm. casein) to dissolve it. The lard was melted and incorporated into the casein solution with a mechanical stirrer. The starch was dissolved in boiling water and it, along with the salt mixture, was added to the protein-fat emulsion. The sucrose and vitamins were added last, with enough water to lower the dry matter content to 19%. The entire mixture was then strained, and run through a homogenizer under a pressure of 2500 lb. The pigs were hand-fed, eight times daily, from a bottle equipped with a rubber nipple. The diet was warmed to about 40°C. at each feeding. The pigs were quartered in small pens, on wooden floors, with shavings for bedding. It is essential to keep young pigs warm; therefore the temperature of the room was kept at approximately 75°F. A few pigs were retained past the age when they would normally be weaned, and when they weighed 25-30 lb. they were given their rations in dry form. Undoubtedly the transfer could be effected much sooner. For convenience the results will be presented in two sections.

*Adequacy of diets which contain no water-soluble vitamins  
except the synthetic compounds*

The mortality rate was rather high during the first few days the pigs received the experimental diets. In all probability many of the deaths were due to the fact that the diet is partially inadequate, but in most cases the early deaths were precipitated by failure of the pigs to consume the diet. Animals will not consume normal amounts of a deficient diet after the deficiency becomes effective. When it first became apparent that diet 308 is inadequate the four water-soluble vitamins it does not contain were added, singly or in combination. The more significant data are summarized in table 2. The essential details are brought out more clearly in figure 1.

The animals shown in table 2 are grouped according to the number of vitamins in the diet, and according to the dates when they were under observation. However, a considerable number of the animals in group IIa were started without adding one at the same time to group IIb. There were thirty-four animals in all and fifteen of them died, though a few of the deaths occurred after the close of the experimental period. Most of the others made slow gains in weight until they were 4 or 5 weeks old.

Of the pigs in group Ia, all under observation at the same time, one made practically no gain in weight and died. Another made moderate gains in weight and was normal in appearance, but was dropped from the experiment after 25 days to conserve food. The third pig gained at somewhat less than the normal rate for 18 weeks when the experimental feeding was discontinued. The hair and skin were noticeably coarse and rough but there were no other signs of malnutrition and no reason to suppose the pig would not attain a more or less normal maturity. Despite the good record made by this pig it is certain that rations such as no. 308 are partially inadequate during the first few days or weeks of a pig's life. The higher degree of success attained

TABLE 2

*Diets which contain no water-soluble vitamins except synthetic compounds are inadequate.*

GROUP	ADDITIONS TO RATION 308	NO. OF PIGS	EXPERI- MENTAL PERIOD	INITIAL WEIGHT	AVERAGE DAILY GAIN
			days	lb.	lb.
I	a	1	128	3.7	0.56
		1	25	3.1	0.30 normal
		1	19	3.0	0.04
	b	1	26	3.0	0.35
		4	12-26	3.0	0.19
	a	1	89	4.0	0.18
II		20	10-58	3.0	0.07
	b	5	3-11	2.7	0.09
					ascorbic acid, biotin, in- ositol, p-amino- benzoic acid

by Hughes ('40) is probably due to the fact that he began with pigs which were much older and which had intestinal flora of a different type, and the intestinal bacteria were able to synthesize the unrecognized vitamins required by swine.

A preliminary trial had given some indication that p-aminobenzoic acid would improve ration 308, and for that reason it was investigated more intensively. Subsequent studies indicated that the ration was not improved by including this compound (Ib). Later on the other available vitamins, or substances reported to be vitamins, were included. The amounts added in mg. per 100 gm. of diet were, respectively, ascorbic acid 30, inositol 30, p-aminobenzoic acid 30, and biotin 0.03.

Five pigs received ration 308 plus these four substances, but there was no evidence that the added vitamins improved the diet in any respect. Other pigs, which will not be described, received one, two, or three of these vitamins, in addition to ration 308, but as would be expected the results were no better than when all four were supplied together. It was concluded that the pig either does not require any of these four vitamins, or else an unrecognized vitamin was the first limiting factor.

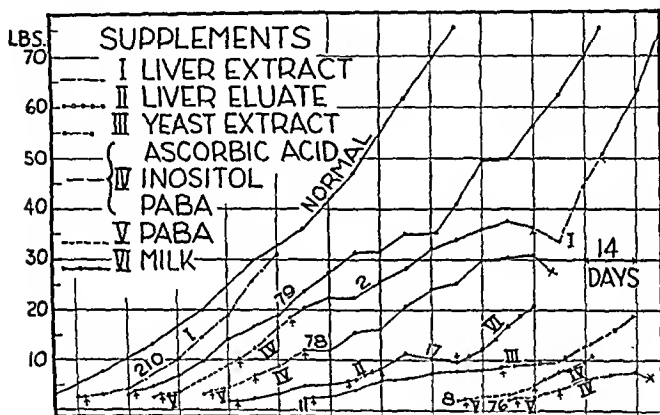


Fig. 1 As a rule pigs grow slowly or fail on ration 308, indicated by an unbroken line. Pig 79 is an exception. Pigs grow normally if diet 308 is fortified with liver or yeast extracts (pigs 2, 210, and 11). A short trial indicated that the active agent of liver extract can be adsorbed on fuller's earth and eluted (pig 17). Ration 308 is not improved by the addition of ascorbic acid, inositol, or p-aminobenzoic acid (pigs 2, 76, 78).

As was mentioned before it has been shown that vitamins A and D, and thiamine, riboflavin, pyridoxine, pantothenic acid, and nicotinic acid are required by swine. Decisive data on the requirement of this animal for choline and vitamins E and K are not available, and an attempt was made to determine whether these vitamins are required by the pig. The experimental diet was the same as ration 308 except it did not contain these three vitamins. Since the results were negative the data will not be reported in detail. It is sufficient to say that the pigs made very slight gains in weight but they gained as rapidly as did the controls, and there were no specific symptoms of any deficiency.



The data do not indicate that the pig requires any of these three vitamins. This interpretation is well supported since satisfactory rations which contain no vitamin E, vitamin K, or choline were described by Hughes and Ittner ('42). Wintrobe and coworkers ('42) reared normal pigs on rations which presumably contained neither vitamin E nor vitamin K.

The characteristic symptoms of pigs fed the basal diet, ration 308, are as follows. All pigs developed rather severe diarrhoea within 24 hours after they were placed on experiment, and some of them never recovered from this disorder. However, after about a week, most of the surviving animals did recover and a few grew fairly well for as long as 2 to 5 weeks. Following this time their feed consumption was irregular, the diarrhoea returned, they became rough and unthrifty, and the rate of growth declined. Some of the pigs developed a sticky, dirty exudate around the eyes and mouth and on the inside of their legs. If the diet was not changed the symptoms became more and more severe, and the pigs eventually died. Post mortem examination failed to disclose any characteristic abnormalities. The liver and kidneys of all the pigs fed ration 308 appeared to be normal. Pneumonia was the immediate cause of death in a few of the pigs that died within the first 3 or 4 weeks after they were placed on experiment. Peritonitis was the immediate cause of death of some of the pigs which survived for 1 or 2 months. Necrotic and inflamed areas were found in the intestinal tract of a few animals.

*Adequacy of diets which contain crude vitamin carriers as sources of the unrecognized vitamins required by swine*

After some of the pigs on ration 308, or one of its modifications, had begun to decline and it seemed certain that these animals require a factor or factors in addition to the vitamins now recognized, a variety of crude vitamin carriers was added to the diet. This was done to show that the failures were not due to disease and that the pigs would grow if the ration was adequate. Furthermore it would be helpful in future work to have data concerning crude vitamin carriers that are good sources of the missing factor or factors. The effectiveness of these crude preparations was usually tested by the curative method, but in a few cases they were supplied from the beginning. Extracts of yeast and liver were used most extensively, and they are described below.

A. An aqueous extract of brewer's yeast concentrated to contain 50% dry matter and centrifuged to remove suspended solids.

B. An aqueous extract of beef liver concentrated to a low volume and treated with an equal volume of 95% ethanol. The filtrate was concentrated to a syrup (O'Dell and Hogan, '43).

C. An aqueous extract of fresh pork liver concentrated to yield a dry matter content of 30-50%.

D. An alcoholic extract of beef liver, similar to fraction 4303 of Hogan, Richardson, Patrick, and Kempster ('41).

E. An aqueous extract of beef liver, similar to fraction 4080 of Hogan, Richardson, Patrick, and Kempster ('41).

F. An aqueous extract of beef liver adsorbed with fuller's earth at pH 1, and the adsorbate eluted with 0.2 N ammonium hydroxide. This preparation is the fuller's earth eluate of Richardson, Hogan, and Karrasch ('42).

Whenever an extract was included in a ration it replaced sucrose. All replacements and calculations are based on dry weight. The data obtained with these extracts are shown in table 3. Each record is for an individual animal.

It will be observed in table 3 that the yeast extract, A, was moderately effective in increasing the growth rate of pigs which had developed symptoms of malnutrition while consuming ration 308 or some ineffective modification. The response of the pigs which received liver extracts gave the impression that these preparations were more potent than was the yeast extract. However, when pigs consumed either one the diarrhoea disappeared, the food intake and rate of growth increased, and there was marked improvement in appearance. The activity of fraction F, the eluate of a fuller's earth adsorbate of liver extract, is worthy of special mention. Since the supply was limited it was supplied for 9 days only, but after it was included in the diet the rate of gain immediately increased. The change in the appearance of the pig was far more striking than the change in weight. It was a sick animal before the change was made but 9 days later the pig was apparently normal. Two weeks after the feeding of the eluate was discontinued, however, it had a poor appetite, had developed severe diarrhoea, and was becoming emaciated. It recovered again, and began to grow normally on being transferred to a diet of milk. Three pigs received the combination of extracts D and E from the time they were 2 days old. They grew as well, and were as normal in appearance as were the control pigs which received fortified cow's milk. The results with liver extracts provide additional evidence that young pigs require an unidentified nutritional factor. Some additional details,

and a normal growth curve (Ittner and Hughes, 38), are shown in figure 1.

It seems worthy of mention that cow's milk, at least some samples, is slightly deficient for the pig during the first few weeks of life. The symptoms noted were weakness in the hind legs, an exudate around the eyes, a rash on the flanks, and a rough hair coat. However, the pigs regained a normal appearance by the time they were 8 weeks old, and were as heavy as their litter mates that had been nursed by their mothers.

TABLE 3

*Extracts of yeast and liver as sources of unrecognized vitamins required by the pig.*

EXTRACT Type	AMOUNT IN DIET	EXPERI- MENTAL PERIOD	INITIAL		DAILY GAIN BEFORE AND AFTER EXTRACT WAS SUPPLIED	
			Age	Weight	Before	After
	%	days	days	lb.	lb.	lb.
A: brewers' yeast, aqueous .....	5	20	31	4.9	0.06	0.32
	5	36	54	8.7	0.12	0.26
B: beef liver, aqueous treated with 95% ethanol; filtered; filtrate used .....	5	21	106	37.9	0.15	0.62
C: pork liver, aqueous; conc. to give 30- 50% dry matter .....	5	20	61	10.3	0.11	0.32
	5	20	40	7.7	0.12	0.44
D: beef liver, alcoholic extract similar to fraction 4303 (Hogan et al., '41)....	5	35	21	4.3	0.06	0.24
D: .....	2.5	54	2	3.2	...	0.53
E: beef liver, aqueous extract similar to fraction 4080 (Hogan et al., '41) .....	5	30	2	2.7	...	0.31
	5	30	2	2.5	...	0.32
F: beef liver, aqueous, fuller's earth adsorbate eluted with 0.2 N am- monium hydroxide; same as ful- ler's earth eluate of Richardson et al., '42 .....	1	9	33	6.0	0.15	0.30
Entire diet fortified <sup>1</sup> cow's milk .....		51	5	2.3	...	0.56
		51	5	2.5	...	0.47

<sup>1</sup> One liter whole milk + 50 gm. whole milk powder, + 50 mg. iron + 4 mg. copper + 5 mg. manganese + 1.5 mg. iodine.

## DISCUSSION

The nutritional requirements of swine are of great practical importance, but our chief interest at present is in comparing these requirements with those of other species. In this respect animals may for the present be divided into two groups. (1) The mouse, rat, and hamster grow normally on diets which contain no water-soluble vit-

amins except those now recognized. (2) The guinea pig, rabbit, chick, and pig do not grow normally unless their diets include one or more unrecognized vitamins. In the absence of precise information, it is safer to assume, when estimating the adequacy of a diet, that man belongs in this second group. There are excellent reasons for believing that these animals do not all require the same unrecognized vitamins, but it is of practical interest that some of the more effective vitamin carriers contain all unrecognized vitamins of which we have any knowledge. For example they are all present in a water extract of fresh liver.

### SUMMARY

1. Pigs seldom grow normally, and few survive, if they are transferred at 2 days of age to simplified diets that contain no water-soluble vitamins except those now recognized. Occasionally, however, a pig will grow fairly well on such a diet.

2. No evidence was obtained that the pig requires vitamin E, vitamin K, choline, biotin, inositol, or p-aminobenzoic acid, but it may be impossible to reach a final decision in this matter until all essential unrecognized vitamins are available and have been tested.

3. Water extracts of liver or yeast contain all unrecognized vitamins required by the pig.

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# STUDIES OF UNIDENTIFIED VITAMINS REQUIRED BY THE CHICK

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Bauernfeind, Sehumacher, Hodson, Norris and Hewes presented evidence of a new chick growth factor which they believed identical with any of the known vitamins. Sehumacher and Norris ('40) showed that the new growth factor of B<sub>12</sub> associates ('38) was a complex consisting of at least two factors which they designated factor R and factor S. Record and Norris ('40) presented evidence indicating that one of the factors, factor S, and associates ('40) might be choline.

In further studies on factors R and S of Sehumacher and Norris ('40), evidence has been obtained which confirms the identity of these factors and shows that neither one of them is choline, folic acid or vitamin B<sub>12</sub>. If folic acid, the Lactoflavin factor, and vitamin B<sub>12</sub> are the same substance, as the results of Pfaffner and associates ('43), Stockstad ('43) and Mitchell ('44) indicate, a second chick antianemic factor, distinct from factor R and S, has been revealed. The results of these studies are presented in this report.

## EXPERIMENTAL

### *Preparation of factor R and S concentrates*

Strain S dried brewers' yeast<sup>2</sup> was used in the preparation of factor R and S. Three methods of extraction were employed. (1) Water extracts were prepared by heat extraction of 5 kilograms of yeast in 25 liters of water for 1 hour with constant stirring. The insoluble residue was filtered and reextracted under the same conditions. (2) Acid extracts were prepared by the same procedure using 0.1N hydrochloric acid extraction mixture was adjusted to pH 6.5 with 40% sodium hydroxide solution. (3) Digestion extracts were prepared by

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yeast with takadiastase.<sup>3</sup> One kilogram of yeast was suspended in 10 liters of water, 10 gm. of takadiastase were added and the pH was adjusted to 4.5 with acetic acid. The mixture was incubated for 24 hours at 37°C. under benzene, brought to pH 6.5 with NaOH, steamed for 30 minutes, cooled and filtered. The insoluble residue was washed by re-suspension in 10 liters of water and filtered.

The combined filtrates from each extraction of yeast were concentrated under reduced pressure (40–50 mm. Hg). The syrup was brought to pH 1.6 by addition of concentrated HCl and the volume was adjusted so that 1 ml. was equivalent to 2 gm. of original yeast. To the acidified concentrate 10 volumes of 95% ethanol were added slowly with constant stirring. Stirring was continued for 30 minutes and the mixture was allowed to stand overnight. The precipitate containing factor S (fraction 1), which formed in the acid-alcohol mixture, was removed by filtration. The acid-alcohol filtrate was brought to pH 7.0 by addition of 40% NaOH with constant stirring. The neutral alcohol mixture was allowed to stand overnight and the precipitate containing factor R (fraction 2) which formed was removed by filtration. The neutral alcohol filtrate (fraction 3) containing folic acid but free of factor R and factor S as determined by growth studies, was concentrated under reduced pressure to a small volume in preparation for feeding.

#### *Experiments with simplified diet 543*

A modification of diet 540 of Schumacher, Heuser and Norris ('40) was used in these studies. The composition of this diet, designated simplified diet 543, is given in table 1. The average choline content of this diet was found to be 0.08%. With the added choline it contained 0.18% choline. The extra choline was made necessary by the discovery of Record and Bethke ('42) and by observations made at this laboratory that diet 540 was deficient in this factor. The results of experiments conducted at this laboratory<sup>4</sup> showed that the addition of 0.2% choline to diet 543 was no more effective than 0.1% in promoting growth and preventing perosis in chicks. Hegsted, Mills, Elvehjem and Hart ('41) have reported that the choline requirement of the chick is approximately 0.1%.

Three experiments were conducted to determine the growth activity of the fractions prepared from the different yeast extracts. Fractions 1, 2 and 3 were each fed at levels equivalent to 5, 10 and 15% of yeast,

<sup>3</sup> Parke, Davis and Company, Detroit, Michigan.

<sup>4</sup> Unpublished results.

and in all possible combinations at levels equivalent to 10% of yeast. All lots of each experiment contained fifteen White Leghorn chicks of mixed sex. The summarized results are presented in table 2. The mortality in these experiments was not excessive and was somewhat lower with yeast and fraction 2.

TABLE 1  
Composition of basal diets.

INGREDIENT	DIET 543	DIET 653
	gm.	gm.
Degerminated yellow corn meal	66.65	...
Corn starch	..	51.7
Purified casein	10.00	27.5
Peanut oil meal	15.00	...
Gelatin	....	7.5
Salt mixture	5.00 <sup>1</sup>	5.0 <sup>2</sup>
Soybean oil	3.00	4.5 <sup>3</sup>
Cellophane	..	3.0
Fish liver oil	0.25 <sup>4</sup>	0.5 <sup>4</sup>
Choline chloride	0.10	0.2
Inositol	...	0.1
	mg.	mg.
p-Aminobenzoic acid	..	10.0
Nicotinic acid	..	3.0
Calcium d-pantothenate	0.7	1.63
2-Methyl-1,4-naphthoquinone	..	1.0
Riboflavin	0.5	1.0
$\alpha$ -Tocopherol	..	1.0
Pyridoxine hydrochloride	0.5	0.5
Thiamin chloride	0.3	0.5
Biotin	..	0.01

<sup>1</sup> Described by Schumacher and Heuser ('40).

<sup>2</sup> Bone meal—2270 gm.,  $K_2HPO_4$ —840 gm., pulverized limestone—700 gm., NaCl—600 gm.,  $MgSO_4 \cdot 7H_2O$ —500 gm.,  $Fe_2(SO_4)_3 \cdot xH_2O$ —55 gm.,  $MnSO_4 \cdot 4H_2O$ —29 gm., KI—3.5 gm.,  $CaSO_4 \cdot 5H_2O$ —1.5 gm.,  $ZnCl_2$ —1.0 gm.,  $CoCl_2 \cdot 6H_2O$ —0.2 gm.

<sup>3</sup> Phosphoric acid added to the extent of 0.2% of soybean oil as an antioxidant.

<sup>4</sup> Contained 400 A.O.A.C. units of vitamin D and 2000 U.S.P. units of vitamin A per gram.

The significance of the differences in growth response between the various treatments and between the three experiments was ascertained by means of analysis of variance (Snedecor, '34). No significant growth differences between the three experiments were found. This demonstrated that the methods of yeast extraction did not influence the distribution of growth activity in the various fractions, and that the data from the three experiments could be combined.



The results showed that fraction 1 promoted a growth response significantly greater than that of the unsupplemented control (odds > 88:1), and that growth was not improved by increasing the amount fed. This growth-response plateau, significantly less than the response to yeast (odds > 99:1), demonstrated that fraction 1 contains a growth factor (or factors) required by the chick, but is lacking in at

TABLE 2

*Growth promoting effect of yeast fractions when added to simplified diet 543.<sup>1</sup>*

Experiments 1, 2 and 3.

SUPPLEMENT	AVERAGE FOLIC ACID ADDED	NUMBER CHICKS 4 WEEKS	AVERAGE WEIGHT 4 WEEKS <sup>2</sup>
	<i>μg./100 gm.</i>		<i>gm.</i>
None	0.00	40	169
Fraction 1 $\approx$ 5% yeast	1.68	41	202
Fraction 1 $\approx$ 10% yeast	3.36	40	208
Fraction 1 $\approx$ 15% yeast	5.04	39	209
Fraction 2 $\approx$ 5% yeast	4.28	39	232
Fraction 2 $\approx$ 10% yeast	8.56	41	243
Fraction 2 $\approx$ 15% yeast	12.84	45	257
Fraction 3 $\approx$ 5% yeast	3.97	34	166
Fraction 3 $\approx$ 10% yeast	7.94	38	170
Fraction 3 $\approx$ 15% yeast	11.91	40	170
Fractions 1 + 2 $\approx$ 10% yeast	11.93	44	263
Fractions 2 + 3 $\approx$ 10% yeast	17.17	43	265
Fractions 1 + 3 $\approx$ 10% yeast	11.97	35	199
Fractions 1 + 2 + 3 $\approx$ 10% yeast	20.53	42	262
Yeast <sup>3</sup>	45.20	45	268

<sup>1</sup> Diet contained 26.7  $\mu$ g. folic acid per 100 gm.

<sup>2</sup> Mean of male and female average weights.

<sup>3</sup> Ten per cent yeast used in two experiments. Eight per cent equal in growth-promoting effect to 10%, used in third experiment.

least one additional growth factor. These results agreed with those obtained by Schumacher, Heuser and Norris ('40) using a similar acid-alcohol precipitate fraction designated factor S. Therefore, fraction 1 contains the factor S of those workers.

Fraction 2 promoted a growth response significantly greater than that of fraction 1 (odds > 99:1). As the amount of fraction 2 was increased, the growth response gradually increased until it approximated that promoted by yeast. Since fraction 1 supported only a submaximal

growth-response plateau, fraction 2 contained at least two factors because it was approximately equivalent to yeast in growth-promoting effect. These results differed from those obtained by Schumacher, Heuser and Norris ('40) since they obtained a submaximal growth-response plateau by feeding graded amounts of a neutral-alcohol precipitate prepared in a manner similar to fraction 2. This second factor they designated factor R. Therefore, fraction 2 contained factor R of Schumacher and associates ('40), but was contaminated with a small amount of factor S. Fraction 3, the alcohol-filtrate fraction, did not promote any significant growth response alone or in combination with any of the other fractions. In spite of the difference in growth response with factor R preparations, these experiments confirm the work of Schumacher and associates ('40) showing that at least two unidentified growth factors, factors R and S, are required by the chick.

In other work<sup>4</sup> with diet 543 factor R was freed of factor S by treatment of fraction 2 with Darco G-60. The adsorbed factor R, when graded levels were fed, was then found to give a submaximal growth-response plateau the same as in the work reported by Schumacher, Heuser and Norris ('40). Moreover, the filtrate remaining after treatment with charcoal was found to have a growth-promoting effect when fed at a level equivalent to 10% yeast but failed to supplement fraction 1 when supplied at the same level. Later with a purified diet it was found that fraction 2 is sometimes free of factor S before treatment with activated charcoal.

The folic acid content of the yeast fractions presented in table 2 was determined by the method of Mitchell and Snell ('41) using *Streptococcus lactis* R. The primary standard for the determinations was a partially purified folic acid preparation obtained from Dr. R. J. Williams of the University of Texas. It was stated to be 7.7% as pure as the material reported by Mitchell and Snell ('41). The standard for routine assays was a sample of liver fraction "B",<sup>5</sup> which was standardized against the folic acid concentrate. The samples were prepared for assay by a method similar to that of Cheldelin, Eppright, Snell and Guirard ('42).

The folic acid assay procedure of Mitchell and Snell ('41) was considered to give accurate results only in assays in which the folic acid concentrate and liver fraction "B" produced superimposable growth-response curves, and only when mixtures of these two materials promoted the same growth response as an equivalent amount of folic acid

<sup>4</sup> Unpublished results.

<sup>5</sup> The Wilson Laboratories, Chicago, Illinois.

from either one alone. The galvanometer readings falling outside the straight-line portion of the standard growth curve were not used in establishing folic acid content. This procedure has been found recently at this laboratory<sup>6</sup> to give results in agreement with those obtained by the revised assay procedure reported by Luckey, Briggs and Elvehjem ('44).

The folic acid values presented in the tables of this paper are expressed as micrograms of folic acid of a potency 40,000 times that of the liver fraction "B" used by the University of Texas workers. Under these conditions the liver fraction "B" contained 17.9  $\mu\text{g.}$  of folic acid per gram.

In this work the same type of growth-response curve was obtained with *Streptococcus lactis*, when pure crystalline *Lactobacillus casei* factor prepared from liver by E. L. R. Stokstad<sup>7</sup> was used as a standard, as when Williams' folic acid concentrate or when liver fraction "B" was used. No direct relation was found to exist between the folic acid content of the yeast fractions and their growth promoting activity for the chick. Fraction 3 which contained more folic acid than fraction 1 promoted no growth-response alone or in combination with the other fractions. Therefore, neither factor R nor factor S is identical with folic acid, and the added folic acid, if required, is ineffective in promoting growth in the absence of both factor R and factor S.

However, the folic acid content of simplified diet 543 (26.7  $\mu\text{g.}$  per 100 gm.) was probably adequate to meet the requirements of the chick for this substance. Data obtained in other experiments<sup>8</sup> conducted at this laboratory showed that two appropriately supplemented purified diets, containing 15.2 and 20.0  $\mu\text{g.}$  of folic acid per 100 gm., supported maximum growth under the experimental conditions used. The growth-response in these experiments was not increased by the addition of more folic acid in the form of yeast to the diets.

The report of Pfiffner and associates ('43) that pure preparations of the chick antianemia factor, vitamin B<sub>12</sub>, are active in stimulating the growth of *Lactobacillus casei*  $\epsilon$  and are probably identical with folic acid, made it of interest to determine if chicks fed simplified diet 543 develop anemia. The results showed that the average blood hemoglobin values (9.8 and 9.3 mg. per 100 ml.) of chicks fed the unsupplemented diet were normal and approximately the same as those (9.3 and 9.6 mg. per 100 ml.) of chicks receiving this diet supplemented with yeast. They

<sup>6</sup> See footnote 4, p. 176.

<sup>7</sup> Lederle Laboratories, Pearl River, New York.

<sup>8</sup> See footnote 4, p. 176.

demonstrated that simplified diet 543 used in these studies was not lacking in any antianemic factor, and that neither factor R or S is identical with vitamin B<sub>6</sub> unless more of it is required for maximum growth than for the prevention of anemia.

### *Experiments with purified diet 653*

In order to investigate the properties of factors R and S under more rigorous conditions, studies were conducted using purified diet 653. The composition of the diet is given in table 1. The inclusion of either cystine or calcium gluconate in the diet has been found not to improve chick growth at this laboratory.<sup>\*</sup> Nutritional encephalomalacia, however, was obtained when calcium gluconate was not used. Further difficulty of this character was prevented by adding 0.2% of orthophosphoric acid to the soybean oil in the diet. Whenever this diet was fed alone, growth failure, severe anemia and extreme mortality occurred.

The yeast fractions were prepared from water extracts of yeast in accordance with the procedure described previously. An adsorbate of fraction 2 (fraction 2-A) was fed in these experiments, as well as fraction 1 and fraction 2. A quantity of fraction 2 equivalent to 1 kilogram of yeast was dissolved in 1 liter of water and digested at pH 4.6 with 2 gm. of takadiastase for 24 hours at room temperature. The mixture was brought to pH 7.0 with NaOH and steamed 30 minutes in an autoclave. Upon cooling a flocculent white precipitate formed. This was removed by filtration, dissolved in warm 5% sulfuric acid and reprecipitated with excess ammonia. The filtrate from this precipitation was combined with the original filtrate and brought to pH 3.0 with sulfuric acid. Five gm. of heat-activated Dareo G-60 were added to this mixture. It was then stirred for 30 minutes and filtered.

In the first experiment fraction 2 was fed as a supplement to diet 653 at levels equivalent to 5, 10 and 15% of yeast. Fraction 2-A was also fed at these levels. Each lot contained fifteen White Leghorn male chicks. The results are given in table 3, experiment 4.

The results showed that growth-response plateaus were obtained with levels of fraction 2 and fraction 2-A equivalent to 10% yeast. In both instances the plateaus were significantly lower than the growth-response obtained with yeast. Therefore fraction 2 and fraction 2-A contained a factor (factor R) required for growth and lacked another factor required along with factor R for maximum growth.

<sup>\*</sup> See footnote 4, p. 176.

TABLE 3

*Growth promoting effect of yeast fractions when added to purified diet 653.<sup>1</sup>*

SUPPLEMENT	FOLIC ACID ADDED	NUMBER CHICKS 4 WEEKS	AVERAGE WEIGHT 4 WEEKS	NUMBER CHICKS 6 WEEKS	AVERAGE WEIGHT 6 WEEKS	BLOOD HEMO- GLOBIN 5 WEEKS
	$\mu\text{g.}/100 \text{ gm.}$		$\text{gm.}$		$\text{gm.}$	$\text{gm.}/100 \text{ ml.}$
Experiment 4						
None	0.0	3	92	0	...	4.3 (7) <sup>2</sup>
Fraction 2 $\approx$ 5% yeast	5.0	13	173	11	245	7.2 (11)
Fraction 2 $\approx$ 10% yeast	10.0	15	248	15	440	8.9 (15)
Fraction 2 $\approx$ 15% yeast	15.0	15	243	13	434	9.6 (13)
Fraction 2-A $\approx$ 5% yeast	5.0	13	139	7	200	5.4 (8)
Fraction 2-A $\approx$ 10% yeast	10.0	13	241	12	424	7.9 (12)
Fraction 2-A $\approx$ 15% yeast	15.0	14	252	14	438	9.2 (14)
10% yeast	48.4	15	312	14	548	9.9 (15)
Experiment 5						
None	0.0	7	112	1	150	3.5 (5)
Fraction 1 $\approx$ 5% yeast	4.6	9	137	4	214	5.5 (6)
Fraction 1 $\approx$ 10% yeast	9.2	12	174	9	245	5.1 (11)
Fraction 1 $\approx$ 15% yeast	13.8	14	199	13	261	6.5 (13)
Fraction 2 $\approx$ 15% yeast	15.0	15	206	15	379	9.0 (15)
Fraction 1 + fraction 2 $\approx$ 10% yeast	19.2	15	244	13	491	9.8 (13)
Fraction 2-A $\approx$ 15% yeast	15.0	14	214	14	361	6.8 (14)
Fraction 2-A + fraction 1 $\approx$ 10% yeast	19.2	14	255	14	493	9.0 (14)
Fraction 3 $\approx$ 10% yeast <sup>3</sup>	7.7	4	100	0	...	4.8 (2)
10% yeast	48.4	15	279	14	488	9.2 (14)
Experiment 6 <sup>4</sup>						
None	0.0	8	111	1	130	4.6 (3)
Fraction 2-A $\approx$ 10% yeast	5.0	10	173	9	276	9.5 (9)
Fraction 3-A $\approx$ 10% yeast <sup>5</sup>	6.5	6	108	0	...	4.7 (2)

<sup>1</sup> Diet 653 contained 8.9  $\mu\text{g.}$  folic acid per 100 gm.<sup>2</sup> Hemoglobin at 26 days. Figure in parentheses indicates number of chicks.<sup>3</sup> Darco adsorbate prepared from fraction 2.<sup>4</sup> Also contained fraction 1-F, fraction 1 freed of folic acid and the antianemic factor by treatment with Darco.<sup>5</sup> Each lot contained 10 White Leghorn male chicks at start. Chicks fed a modification of diet 653, containing glucose in place of corn starch, somewhat less casein and gelatin, and 0.5% cystine. The modified diet contained 7.8  $\mu\text{g.}$  folic acid per 100 gm.<sup>6</sup> Darco adsorbate prepared from fraction 3.

A further study was conducted to determine the supplementary action of factor S present in fraction 1, and factor R present in fractions 2 and 2-A when included in purified diet 653. Fraction 1 was fed at levels equivalent to 5, 10 and 15% of yeast. The same preparations of fractions 2 and 2-A, used in experiment 4, were fed at levels equivalent to 15% of yeast, in order to establish the maximum growth-responses of the chicks used in this experiment to these materials. Fractions 2 and 2-A were also fed in combination with fraction 1 at levels equivalent to 10% of yeast. Each lot contained fifteen White Leghorn male chicks. The results are given in table 3, experiment 5.

The results showed that fraction 1 produced a growth response which tended to give a plateau at a submaximal level as in previous work. Fractions 2 and 2-A promoted submaximal growth which, in view of the results of experiment 4, represented the maximum responses to these materials. The combination of fraction 1 with fraction 2 or fraction 2-A promoted maximum growth equal to that promoted by yeast. The plateau and supplementary effects obtained in this experiment and the preceding one showed that fraction 1 contains a factor (factor S) which is not identical with factor R present in fraction 2.

The results of the experiments with purified diet 653 confirm the results obtained with simplified diet 543 and demonstrate the existence of two unknown factors, designated factors R and S, which are required for chick growth. Neither one of these factors is choline, since purified diet 653 was adequately supplemented with choline. Also neither one is identical with folic acid since both fraction 1 and fraction 2 promoted submaximal growth-response plateaus upon feeding graded levels in spite of the presence in them of approximately the same quantities of folic acid. If one of the factors had been folic acid, a submaximal plateau would have been revealed in one instance only.

In these experiments both fraction 1 and fraction 2 were found to possess antianemic properties. Fraction 1, however, was much less effective in preventing anemia than fraction 2 in spite of the fact that it contained almost as much folic acid as this fraction. The addition of 13.8  $\mu$ g. of folic acid per 100 gm. of diet 653 by means of fraction 1 maintained a hemoglobin level of only 6.5 gm. per 100 ml. of blood as compared to a level of 8.9 gm. per 100 ml. maintained by the addition of 10  $\mu$ g. of folic acid by means of fraction 2. Fraction 1, therefore, is deficient in an antianemic factor present in fraction 2, and this antianemic factor is not folic acid. The somewhat higher hemoglobin level maintained by fraction 1 as compared to the unsupplemented purified diet probably was due, in part at least, to contamination of factor S

with this antianemic factor, since preliminary results showed that Darco treated fraction 1 alone possessed no antianemic properties. It was not entirely due to folic acid since in experiment 5 the addition of fraction 3, which contains folic acid but no factor R or S, to the purified diet failed to increase the hemoglobin level. The same results were obtained in experiment 6 with fraction 3-A, the adsorbate of fraction 3. On the other hand, fraction 2-A, the adsorbate of fraction 2, which contained less folic acid in this experiment than fraction 3-A promoted normal hemoglobin formation.

The fraction 2 and fraction 2-A, used in experiments 4 and 5, were somewhat more effective in promoting growth than in preventing anemia. Both fractions at levels equivalent to 10% yeast promoted growth as great as that obtained at the 15% levels but the 15% levels of these fractions were better than the 10% levels in preventing anemia. Furthermore, in both experiments the adsorbate of fraction 2 appeared somewhat less effective in promoting hemoglobin formation than fraction 2 but no evidence was obtained of an accompanying reduction in growth promoting effect. This decrease in antianemic effect was particularly striking in experiment 5 where chicks of somewhat lower inherent growth capacity were used. At times, however, these differences were not observed, as in experiment 6, when the yeast extract used in preparing the fraction was richer in the antianemic factor than that from which the fractions studied in experiments 4 and 5 were prepared. In spite of this the results of the experimental work with purified diet 653 indicate that the antianemic factor is not identical with factor R, and that it is either somewhat less readily adsorbed by activated charcoal or less readily eluted by the chick than the growth factor. It is evident also that the antianemic factor is not identical with factor S.

These conclusions are strengthened by comparing the results obtained with purified diet 653 with those obtained with simplified diet 543. The latter diet was found to be deficient in factors R and S but not in the antianemic factor or folic acid. Purified diet 653, on the other hand, was found to be lacking in factors R and S and the antianemic factor. It also may be deficient in folic acid. With simplified diet 543 both factor R and factor S were shown not to be identical with the antianemic factor unless more of these factors are required for maximum growth under the experimental conditions than for the prevention of anemia. This is impossible since the work with purified diet 653 demonstrated that the antianemic factor accompanies factor R for the most part and that in some instances more of fraction 2 and fraction 2-A which contain factor R was required to prevent anemia under the experimental conditions than to promote maximum growth.

## DISCUSSION

According to the work of Mitchell, Snell and Williams ('41), Hutchings, Bohonos and Peterson ('41), Stokstad ('43), and Mitchell and Williams ('44), folic acid and the *Lactobacillus casei* factor appear to be identical. The work of Piffner and associates ('43) and Stokstad ('43) provides evidence that the *Lactobacillus casei* factor and vitamin B<sub>12</sub> are also identical. If those factors are the same, the antianemic factor encountered in the experiments reported in this paper is a new factor, differing from the substance variously designated folic acid, *Lactobacillus casei* factor and vitamin B<sub>12</sub>.

The work conducted with purified diet 653, however, does not preclude the possibility that folic acid is a factor required by the chick both for growth and the prevention of anemia. When fraction 3 or fraction 3-A only were added to purified diet 653 the hemoglobin level was not increased. Growth failure and 100% mortality also occurred as was observed in several instances with the purified diet alone. However, folic acid may function as an antianemic and growth factor only in the presence of the antianemic factor found chiefly in fraction 2. The existence of a second antianemic factor is suggested by the work of O'Dell and Hogan ('43). If this is true, the folic acid requirement of the chick for growth and the prevention of anemia appears to be less than 15 µg. per 100 gm. of diet. Briggs, Luckey, Elvehjem and Hart ('43) have reported maximum growth in chicks fed a purified diet containing 17.5 µg. of added folic acid per 100 gm. The folic acid content of the purified diet was not given but, since it was similar to purified diet 653, it probably contained approximately 8 µg. per 100 gm.

The evidence that the chick requires a second antianemic factor in addition to folic acid, however, is difficult to reconcile with the work of Piffner and associates ('43) and Campbell, Brown and Emmett ('44). Under their experimental conditions, these workers obtained maximum growth in chicks and prevention of anemia by supplying from 100 to 400 µg. of vitamin B<sub>12</sub> per 100 gm. of diet. The antianemic effect obtained by Campbell and associates ('44), however, was not as striking as that obtained by Piffner and associates ('43) and may have been subnormal. In contrast to these growth results, Almquist ('43) failed to obtain any significant growth increase by supplying 150 µg. of *Lactobacillus casei* factor per 100 gm. of diet by means of a concentrate, approximately 9% pure, prepared by Stokstad.<sup>10</sup>

In view of these results and in the light of the studies reported in this paper showing that the folic acid requirement of the chick is less

<sup>10</sup> See footnote 7, p. 180.



than 15  $\mu$ g. per 100 gm. of diet, it is suggested that the antianemic effect of crystalline vitamin B<sub>6</sub> and also its growth promoting properties are caused either by contamination with extremely potent antianemic and growth factors or to synthesis of these factors by bacteria in the intestinal tract as a consequence of supplying large amounts of a necessary bacterial growth factor. O'Dell and Hogan ('43) showed that the incidence of anemia is increased by including sulfaguanidine in the purified diet fed the chicks. If either of these explanations proves to be correct, then the antianemic factor encountered in the studies described in this report is probably the vitamin originally discovered by Hogan and Parrott ('40) and designated vitamin B<sub>6</sub>.

In view of the ease of adsorption by activated charcoal and because of similarities in source and function, factor R may be identical with factor U of Stokstad and Manning ('38). Vitamin B<sub>11</sub> of Briggs, Luckey, Elvehjem and Hart ('43) appears to be the same as factor R of Schumacher, Heuser and Norris ('40) but is associated with an antianemic factor. The other unidentified factor suggested by Briggs and associates ('43) as a consequence of failure to obtain as good growth with a biotin concentrate as with kidney residue may be identical with factor S of Schumacher and associates ('40).

No evidence of the existence or non-existence of a factor essential for feather development only was obtained in the experiments reported in this paper. Factor S, however, did not promote as good feather development as factor R. Since the results of Briggs, Luckey, Elvehjem and Hart ('43) indicate that there is a feather factor distinct from vitamin B<sub>11</sub>, it may accompany factor R in fraction 2.

#### SUMMARY

Evidence, obtained previously, of the existence of two unidentified chick growth factors, designated factor R and factor S, has been confirmed. Factors R and S have been found not to be identical with folic acid, the *Lactobacillus casei* factor or vitamin B<sub>6</sub>. If the latter factors are the same substance, as recent work indicates, a new chick antianemic factor has been revealed which is distinct from factors R and S. This antianemic factor may be vitamin B<sub>6</sub>, however, in the event that crystalline preparations of it are contaminated with highly potent growth and antianemic factors or stimulate their bacterial synthesis in the intestinal tract. No evidence was obtained that folic acid is, or is not, required by the chick, since growth failure and very extreme mortality occurred in the absence of the antianemic factor and factor R or

the antianemic factor and factor S. If folie acid is required, the amount appears to be less than 15  $\mu$ g. per 100 gm. of diet.

#### ACKNOWLEDGMENT

We are indebted to Dr. E. L. R. Stokstad of Lederle Laboratories, Pearl River, New York, for pure *Laetobacillus casei* factor, to Dr. Y. Subbarow of Lederle Laboratories for biotin concentrates, to Dr. R. J. Williams, University of Texas, for standardized folie acid, and to Anheuser-Busch, St. Louis, Missouri, for Strain S dried brewers' yeast.

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# PROTEIN INTAKE AND HEAT PRODUCTION<sup>1</sup>

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During recent years the authors and their associates have conducted six experiments, five with growing and one with mature albino rats, for the purpose of determining the influence of the protein content of equalcaloric diets on the heat production under conditions representing normal nutritive practice; and in these experiments the heat production diminished, at moderate rates, in the increasing order of the intake of protein—which is not in harmony with the generally prevailing understanding that the protein content of diets dominates the heat production by virtue of the high specific dynamic effect of this nutrient as fed alone. Five of these experiments have been published in four papers (Forbes, Swift, Black and Kahlenberg, '35; Forbes, Voris, Bratzler and Wainio, '38; Forbes, Black, Thacker and Swift, '39, '40), and the results of the sixth are presented herewith. In this general program 231 albino rats were used as subjects, with 10 to 12 animals on each dietary treatment, the program as a whole covering 16,170 24-hour "rat days" of controlled experimentation.

In this series of experiments the heat production was measured as single values for 70-day periods by subtracting the caloric equivalents of the excreta and of the body gain from the gross energy of the food. The special advantages of this procedure, which the authors have called "the body balance method", are that the long period of observation serves to minimize the factor of error of work, and covers the complete process of food utilization during a protracted interval. Also, in order that the results shall represent the normal use of food, the animals were fed at super-maintenance planes of nutrition on diets which were nutritively complete, except as influenced by the protein intake, and were allowed unrestricted freedom of motion. This procedure has led to conclusions at variance with the generally prevailing ideas as to the influence of the protein intake on the production of heat because of

<sup>1</sup> Authorized for publication on May 3, 1944, as paper no. 1234 in the *Journal Series of the Pennsylvania Agricultural Experiment Station*.

differences in methods of experimentation, and in the interpretation of results, as will later appear.

To assist in clarifying our understanding of the influence of protein on the heat production from normal, mixed diets the authors conducted the experiment discussed in this paper. The general procedure was the same as in the previous experiments of this series, except that the environmental temperature for the animals was maintained during the entire 70 days of the experiment definitely within the zone of thermal neutrality, that is, within the range of temperature at which no extra nutriment is katabolized for the maintenance of the normal temperature of the body. During the earlier experiments of this series the environmental temperatures were not so controlled and were often slightly below the critical point, though all groups of animals received the same treatment.

*The idea that the dynamic effects of normal diets are in harmony with their protein contents depends primarily on two misconceptions, (1) that specific dynamic effects of nutrients determined at planes of nutrition involving the katabolism of body substance apply to super-maintenance planes of nutrition, and (2) that the specific dynamic effects of individual nutrients, determined by any procedure, are true of these nutrients when combined as in the diets of normal nutrition.*

The usual method of determining the specific dynamic effects of nutrients is to give a single feeding of the test substance to an animal in a post-absorptive state and then, during intervals of time in which the subject is quiet, to measure the maximum height of the increase in heat production, or more properly the total increase in heat production above the base rate, or above a mean value between the initial base rate and a final rate computed to represent the decreased heat production of the subject if it had continued without food during a time interval equal to the period of measurement of the increased metabolism.

The element of experimental error in any such short-time measurements is apt to be large, especially as the result of a technic inadequate for the determination of either the maximum height of the metabolism attained or the total increase in heat production; and it is the authors' understanding that any such measurement, involving the katabolism of body substance, is too low to apply to planes of nutrition at or above maintenance by an amount representing the dynamic effect of the body nutrients katabolized. The point of view from which this observation is made has already been discussed (Forbes and Swift, '41).

That determinations of dynamic effects by procedures implying the heat production at a sub-maintenance status as a base value do not

apply to normal nutrition at super-maintenance rates is also indicated by the fact that the proportion in which the nutrients are utilized is much more prominently affected when the base value is below than when it is at or above maintenance. Also, the assumption that the specific dynamic effects of individual nutrients as determined by any procedure apply to combinations of nutrients is not in accord with established facts.

In connection with the presentation of evidence on the heat increments of diets balanced and unbalanced with respect to protein, Hamilton ('35, '37, '39) has summarized abundant literature showing that nutritive balance, in general, affects the economy of utilization of food energy; and an extensive series of papers from this institute, by E. B. Forbes and associates, on individual nutritive deficiencies as affecting the utilization of food energy, similarly imply that the heat increments and the energy values of nutrients in combination can not be the additive separate heat increments and energy values of these constituent nutrients.

Further, Forbes, Bratzler and associates ('39), Ring ('42), and Forbes and Swift ('44) have conducted experiments showing that the dynamic effects of protein, carbohydrate and fat as individually determined are not true of these nutrients variously combined.

#### EXPERIMENTAL

In the present experiment the plan of the diets as given in table 1 shows that their protein contents were determined by the proportions of beef muscle protein included; and the gross energy values of the diets were rendered equal by compensating adjustment of their contents of carbohydrate<sup>2</sup> and fat.<sup>3</sup> As a result, the arrangement of the diets in the order of their increasing protein contents had the effect of arranging them in the order of their decreasing contents of carbohydrate and fat. Critically speaking, therefore, the differences in the results produced by these diets should be ascribed not to the differences in protein intake alone but to this factor and the associated, compensating differences in carbohydrate and fat.

Referring to table 2—the subjects of this experiment were eleven litter-triplets of weanling, male, rats divided into three groups of approximately the same weight, which received equicaloric quantities of diets containing approximately 10%, 25%, and 45% of protein. All results of this experiment, therefore, are averages representing eleven animals.

<sup>2</sup> Cerelese.

<sup>3</sup> Crisco.

Since the 25% protein diet was more nearly the optimum than the other two, for the growing rat, the gains in live weight and in nitrogen produced by this diet were greater than by the diets of 10% and 45% protein; but the gain in fat decreased in the increasing order of their contents of protein, which was in the decreasing order of their contents of metabolizable energy.

TABLE 1  
*Composition of diets.*

INGREDIENT	DIET 1	DIET 2	DIET 3
	%	%	%
Cellu flour	4.00	4.00	4.00
Salt mixture <sup>1</sup>	4.00	4.00	4.00
NaCl	1.00	1.00	1.00
Yeast <sup>2</sup>	6.00	6.00	6.00
Butterfat	1.00	1.00	1.00
Cerelose <sup>3</sup>	55.60	43.45	27.26
Beef muscle	9.20	29.58	56.74
Crisco	19.20	10.97	0.00
Total	100.00	100.00	100.00

<sup>1</sup> Osborne, T. B., and L. B. Mendel, '17. J. Biol. Chem., vol. 32, p. 369.

<sup>2</sup> The yeast used was a mixture of 5 parts by weight brewer's yeast and 1 part irradiated yeast.

<sup>3</sup> Cerelose is a crystalline dextrose of high purity.

TABLE 2  
*Average amounts of food eaten, and of gains in live weight, nitrogen and fat per rat during 70 days.*

PROTEIN IN DIET	FOOD EATEN, DRY MATTER	LIVE WEIGHT		GAIN IN EMPTY BODY WEIGHT	NITROGEN OF BODY GAIN	FAT GAINED
		Initial	Final			
%	gm.	gm.	gm.	gm.	gm.	gm.
10.06	498.4	33.5	146.2	108.1	3.02	21.68
24.81	499.9	33.1	201.5	161.7	5.38	19.01
44.88	499.8	33.3	180.2	141.1	4.76	16.43

The distribution of the total food nitrogen per rat, for 70 days, is given in table 3; and the distribution of the food energy for the same period is presented, as from experiment no. VI in table 4, which gives parallel data from the entire series of six similar experiments conducted at this laboratory.

The protein variant in experiments I, II and III was casein; and in experiments IV, V and VI it was beef muscle protein.

As revealed by their live weights, the rats in experiments I, II, III, IV and VI were weanlings, in contrast to those in experiment V which

were mature. The lightness of the initial live weights of the rats in experiment VI was due to a nutritive deficiency resulting from war-time restrictions affecting the manufacture of the commercial animal feed which served as the principal part of the stock colony ration.

In this table the data representing "metabolized energy" are the values for food energy minus the energy of the feces and urine, and those for "metabolizable energy" are the values for food energy minus the energy of the excreta and the non-metabolizable energy of the protein retained.

TABLE 3

*Distribution of average 70 days' food nitrogen per rat as related to the plane of protein intake.*

Protein in the diet, %	10.06	24.81	44.88
Nitrogen:			
Food, gm.	8.5	21.0	38.0
Feces, gm.	1.0	1.6	2.7
Digested, gm.	7.5	19.4	35.3
% of food N	88.2	92.4	92.9
Urine, gm.	4.4	13.0	29.4
% of food N	51.8	66.2	77.4
Gain of N, gm.	3.0	5.4	4.8
Calories in urine per gm. urinary N	11.7	8.0	7.0

### CONCLUSIONS

From a series of six experiments, five of which have been previously published, on the utilization of the energy and the protein of equicaloric diets differing in protein content, the following conclusions are drawn:

The apparent digestibility of food nitrogen, and the per cent of the food nitrogen eliminated in the urine increased, and the energy per gram of nitrogen in the urine decreased, in the increasing order of the protein contents of the diets.

The apparently digestible energy, as indicated in reverse by the energy of the feces, increased in the increasing order of the protein contents of the diets at the lower planes of protein intake, but in three experiments among the four in which the diet contained 45% of protein, the plane of maximum apparent digestibility of protein was exceeded.

In all experiments the outgo of energy in the urine increased throughout the entire range of increase in protein intake.

The body gain of energy increased in the increasing order of the protein contents of the diets from 10% to 25%, but decreased in the order of the further increase in protein content of the diets to 45%.



TABLE 4

*Distribution of average 70-day food energy of albino rats related to the percentage of protein in equicaloric diets.*

EXPERIMENT NO. AND REFERENCE TO PUBLICATION	LIVE WEIGHTS OF RATS	PROTEIN IN DIETS, APPROX- IMATE PERCENTAGE	FOOD ENERGY PER RAT	FECES ENERGY	URINE ENERGY	BODY GAIN OF ENERGY	BODY GAIN OF PROTEIN ENERGY	BODY GAIN OF FAT ENERGY	METABO- LIZABLE ENERGY <sup>1</sup>	METABO- LIZABLE ENERGY <sup>1</sup>	HEAT PRODUC- TION
	gm.	%	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.	Cal.
I J. Nutr. 10 (1935), 461	48-126	10	2164	184	47	224	93	131	1933	1914	1703
	48-155	15	2164	178	55	263	137	125	1931	1902	1668
	48-168	20	2164	153	72	296	153	143	1939	1907	1643
	47-167	25	2164	143	93	302	156	145	1928	1895	1626
II J. Nutr. 10 (1935), 461	48-115	10	2076	178	39	226	68	158	1858	1844	1632
	47-142	15	2076	162	54	259	108	152	1860	1837	1601
	48-150	20	2076	148	71	267	117	150	1857	1833	1590
	48-152	25	2076	138	90	270	121	149	1848	1823	1578
III J. Nutr. 15 (1938), 285	47-162	25	2261	161	99	332	129	203	2001	1974	1669
	47-159	30	2261	156	121	320	127	193	1984	1957	1665
	48-159	35	2261	149	142	311	127	184	1970	1943	1658
	47-151	45	2261	140	187	285	119	166	1934	1909	1650
IV J. Nutr. 18 (1939), 47	49-173	10	3136	339	58	364	136	228	2739	2711	2375
	50-218	25	3136	296	142	480	201	279	2698	2656	2218
	50-220	35	3136	279	209	423	208	215	2648	2605	2225
	50-207	45	3136	298	261	415	189	226	2577	2537	2162
V J. Nutr. 20 (1940), 47	385-391	10	4220	498	103	113	9	104	3619	3617	3505
	388-401	25	4220	403	237	124	14	110	3579	3576	3456
	390-388	45	4220	413	401	5	12	-6	3407	3404	3402
VI J. Nutr., (this paper)	34-146	10	2505	275	51	323	116	207	2179	2155	1856
	33-202	25	2505	237	111	387	207	180	2157	2114	1770
	33-180	45	2505	271	206	334	179	155	2028	1991	1694

<sup>1</sup> For a statement of the distinction between metabolized and metabolizable energy see the text.

The body gain of protein energy increased in accord with the increasing protein intake up to the 30% to 35% level, but was less extensive at the 45% than at the 35% level.

The body gains of fat energy represented balances of metabolizable energy supplied in excess of the requirements of the animal for maintenance (including physical activity) and synthesis of protein. The gains in fat energy were not in definite order with reference to the plane of protein intake.

The metabolized energy was about on a level at planes of protein intake up to 25%, but invariably decreased at the higher levels of dietary protein.

The metabolized energy, the metabolizable energy, and the heat production all diminished at about the same slight rate throughout the entire range of increase in the protein contents of the equicalorie diets, thus rendering clear the fact that it was the metabolizable energy, and not the protein content of these diets, which dominated the production of heat.

The improved condition of experimentation provided in the present study as compared with the previous studies of the series — that is, the maintenance of the environmental temperature within the zone of thermal neutrality for the subjects — was without observed effect on the results obtained.

#### SUMMARY

Results are presented of a study of the heat production of growing albino rats in relation to the protein contents of equicalorie diets containing approximately 10%, 25% and 45% of protein; and general conclusions are drawn from six similar studies, including the present experiment, on the same subject.

The special purpose of this last experiment was to determine the effects of maintaining the environment at all times definitely within the zone of thermal neutrality for the subjects. This improvement in conditions of experimentation was without observed effect on the results obtained, as compared with previous findings.

The metabolizable energy and the heat production diminished at about the same slight rate throughout the entire range of increase in the protein contents of the equicalorie diets, thus showing that it was the metabolizable energy, and not the protein content of these diets, which dominated the production of heat.

Other conclusions are given in the text.

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# INTESTINAL ABSORPTION OF GALACTOSE IN THE RAT AS AFFECTED BY SUBOPTIMAL INTAKES OF THIAMINE<sup>1, 2, 3</sup>

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## ONE FIGURE

(Received for publication April 24, 1944)

A decrease in the rate of intestinal absorption of galactose in acute severe thiamine deficiency and in vitamin B complex deficiency has already been reported by this laboratory (Free and Leonards, '42; Leonards and Free, '43b). The possibility that prolonged subacute thiamine deficiency, which is more prevalent than the acute severe deficiency, may have an effect on intestinal absorption led to the present study. Specifically, it was designed to determine the effect of prolonged subsistence on diets low in thiamine on the rate of intestinal absorption of galactose in the rat.

## METHODS

The methods used in the care of animals and in the measurement of intestinal absorption have already been adequately described (Free and Leonards, '42). Three groups of young rats were placed on the vitamin B complex-free diet having the following percentage composition: alcohol-extracted casein 18, starch 68, cottonseed oil 8, Mendel-Hubbell-Wakeman salt mixture ('37), 4, and cod liver oil 2; to 100 parts of this mixture was added 0.2 part of choline chloride. One group of rats received this diet supplemented with 2  $\mu$ g. of thiamine daily, the second group with 5  $\mu$ g. daily and the third group with 10

<sup>1</sup>The material contained in this paper is taken from a dissertation submitted by Jack R. Leonards, to the Graduate School of Western Reserve University, June 1943, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry. The authors are indebted to Prof. Victor O. Myers for making possible the series of investigations on the effect of vitamin deficiencies on intestinal absorption.

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<sup>3</sup>We are indebted to Merck and Co., Rahway, New Jersey, for generous supplies of the vitamins employed in this study.

µg. per day. Each experimental animal was "paired" with a littermate control rat which received 40 µg. of thiamine per day, and whose food intake was limited so that its weight was kept very nearly equal to that of the experimental member of the pair.

All of the rats received the usual adequate daily supplements of riboflavin (25 µg.) pyridoxine (20 µg.), and calcium pantothenate (100 µg.). The animals were kept on this diet for 70 to 80 days at which time the weights of animals receiving the 2 µg. and 5 µg. of thiamine per day had become stabilized. At this time the measurements of intestinal absorption of galactose were carried out.

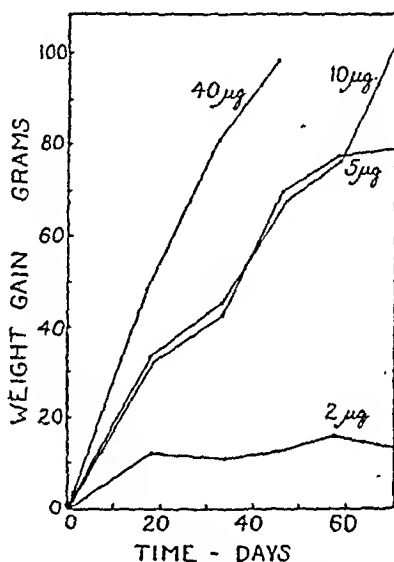


Fig. 1 The effect of various thiamine supplements on the rate of growth of rats.

#### RESULTS

The average growth curves of the rats fed diets containing 2 µg., 5 µg. and 10 µg., respectively, of thiamine daily are given in figure 1, and are compared with those of a group of rats on the same diet eaten ad libitum but supplemented with 40 µg. of thiamine daily. These growth curves do not include the weights of the paired control animals, but as previously mentioned the weights of these controls were kept very nearly the same as those of the experimental companions. The animals receiving 5 µg. and 10 µg. of thiamine daily grew at about the same rate for the first 8 weeks after which time the 5 µg. group did not gain any more weight. Two µg. of thiamine daily was sufficient to enable the rats to attain a weight of about 75 gm. The weights then fluctuated

within 5 or 10 gm. of this figure for the last 50 days of the experiment. Thus the condition of most of the animals in this group was kept rather constant for a considerable period of time. Four of the animals receiving 2  $\mu$ g. of thiamine per day exhibited marked symptoms of polyneuritis after being on the diet for about 60 days. These were promptly cured by allowing the rats to drink a solution containing 20  $\mu$ g. of thiamine. Then, in order to prevent the rats from gaining too much weight, the regular daily thiamine supplements were withheld for 5 or 6 days. This procedure was thought to be preferable to attempting to cure the rats with smaller doses of thiamine. No recurrences of polyneuritis were observed in these rats.

The animals receiving the higher levels of thiamine appeared normal in all respects, except for a slight decrease in the rate of growth. The rats on the 2  $\mu$ g. daily level of thiamine, as well as their pair-fed controls, appeared emaciated and had rough fur. Priapism was observed in many of the animals both in the deficient and control groups. Although care was taken to assure an adequate supply of pantothenic acid, about one-half of the animals in the 2  $\mu$ g. level group and their controls showed a "rusting" of the white fur around the neck, similar to that seen in pantothenic acid deficiency (McElroy, Salomon, Figge and Cowgill, '41). This was only of a very mild degree, and there were no "blood eaked" whiskers.

The individual results of the absorption studies of 25 pairs of rats in which the experimental member was allowed 2  $\mu$ g. of thiamine daily are given in table 1. The average absorption coefficient of the deficient animals was 298 mg. per 100 gm. per hour while that of the controls averaged 354 mg. per 100 gm. per hour. The rate of absorption of the deficient animals therefore averages 85% of that of the controls. This decrease in rate of absorption of the chronically deficient animals, although small and at the borderline of statistical significance, is exhibited by twenty-one of the twenty-five animals that were studied. That this decrease is not due mainly to changes in the factors of gastric motility and emptying time is shown by the fact that the intestines of the deficient animals contained even more galactose than those of the controls. The values are, respectively, 11% and 7% of the ingested amount of the sugar.

The average values for the blood galactose 1 hour after ingestion of the sugar were respectively, 513 mg. per 100 ml. and 578 mg. per 100 ml. for deficient and control groups. Thus the rate of intestinal absorption of the rats on the low thiamine intake, as measured by the blood galactose levels, averaged 89% of that of the control animals.

The blood galactose levels therefore do not indicate any impairment in the rate of metabolism of the sugar as was seen in the case of rats acutely deficient in pantothenic acid and thiamine (Leonards and Free, '43a; Leonards and Free, '43b).

The results obtained on the effects of daily intakes of 5  $\mu$ g. and 10  $\mu$ g. of thiamine daily on the rate of intestinal absorption of galactose are also given in table 1. There is no significant difference in the rate of intestinal absorption in rats receiving 5  $\mu$ g., 10  $\mu$ g., or 40  $\mu$ g. respectively, of thiamine daily. There are, however, two interesting observations in these animals to be reported. One is that the absorption

TABLE 1

*Intestinal absorption in rats receiving different thiamine supplements.*

	NUMBER OF RATS	BODY WEIGHT		GALACTOSE				DIFFERENCE CONTROL MINUS DEFICIENT	
		Initial	Final	In stomach <sup>1</sup>	In intestines <sup>1</sup>	Absorption coefficient	Blood galactose	Absorption coefficient	Blood galactose
		gm.	gm.	%	%	gm./100 gm./hr.	ml.		
2 $\mu$ g. <sup>2</sup>	25	56	71	39	11	298	513		
Control	25	47	74	34	7	354	578	+ 52	+ 61
5 $\mu$ g.	7	45	115	57	10	198	381		
Control	7	39	105	53	7	236	472	+ 38	+ 89
10 $\mu$ g.	8	33	129	53	8	233	444		
Control	8	31	123	49	8	256	478	+ 24	+ 35

<sup>1</sup> Fraction of ingested galactose

<sup>2</sup> Daily thiamine supplement.

coefficients of these groups of animals are much lower than those obtained for all of the other groups of rats in vitamin deficient states which have been studied in this laboratory. The other observation is that the per cent of ingested galactose remaining in the intestines of these rats was also lower than that characteristic of other groups of animals, the amount of sugar in the stomach being correspondingly increased. One reason for these differences may be due to the fact that the growth of these animals was not stunted as much as in the more severe deficiencies.

Harper ('42) studied the effect of suboptimal intake of thiamine on the rate of intestinal absorption in rats. The rats were given a thia-

mine deficient diet for 6 weeks or until their food consumption diminished. They were then given 10  $\mu$ g. of thiamine daily for a period which was not recorded. A decrease in the rate of absorption of glucose was observed in the deficient animals. This decrease amounted to 17% of the normal rate when a 2-hour absorption period was used. The effect of gastric evacuation was not recorded. Although the conditions of Harper's experiments were different than those described in this report, both studies indicate that only small decreases in the rate of intestinal absorption of sugars are observed in mild chronic thiamine deficiency.

#### SUMMARY

1. The rate of intestinal absorption of galactose was measured in 3 groups of rats which were kept for 70 to 80 days on thiamine intakes of 2  $\mu$ g., 5  $\mu$ g., and 10  $\mu$ g. per day, respectively. The absorption was compared with "paired" control animals receiving 40  $\mu$ g. of thiamine per day.

2. The rate of absorption of galactose in rats receiving these suboptimal thiamine intakes averaged 85% to 90% of that of the controls.

3. The methods employed did not indicate any change in the rate of metabolism of galactose as a result of the chronic thiamine deficiency.

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# BIOTIN AND FOLIC ACID DEFICIENCIES IN THE MOUSE

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ONE PLATE (FOUR FIGURES)

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Rats maintained on a purified diet containing the B Complex vitamins — thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid and choline chloride — grow normally (Unna, Richards, and Sampson, '41; Henderson et al., '42). It was soon shown by Black, McKibbin, and Elvehjem ('41) and later by others that sulfaguanidine inhibited the intestinal elaboration of additional growth factors required by the rat. The inclusion of sulfasuxidine in a synthetic diet gave rise to a biotin and folic acid deficiency (Nielsen and Elvehjem, '42), and recently it has been shown that an inositol deficiency may be produced by feeding sulfasuxidine, Nielsen and Black ('44). However, the rat can synthesize sufficient biotin, folic acid and inositol to meet its requirements of these factors if maintained on a synthetic diet devoid of a sulfonamide. It has been shown by Woolley ('40b) that the mouse requires inositol for normal hair formation. We now have evidence that the mouse, unlike the rat, requires biotin and folic acid for normal growth on an artificial ration.

## EXPERIMENTAL

The basal ration had the following percentage composition: alcohol extracted sucrose, 68; alcohol extracted casein, 20; salts IV, 5; corn oil, 5; and cod liver oil, 2. The following B vitamins were incorporated into 100 gm. of the basal diet: thiamine chloride, 0.5 mg.; riboflavin, 1.25 mg.; pyridoxine, 0.5 mg.; calcium pantothenate, 2.2 mg.; nicotinic acid, 5 mg.; choline chloride, 200 mg.; and inositol, 200 mg. The rations were never more than a week old and were stored in an ice box. A special feed cup was used in order to overcome the tendency of mice to scatter the diets. An ointment jar 2 inches in diameter and approximately 2 inches in depth was used as a food container. The jar was filled with ration to within a  $\frac{1}{2}$  inch of the top and a metal disc  $1\frac{1}{4}$  inches

in diameter was placed over the feed. The disc had three holes of  $\frac{1}{2}$  inch in diameter through which all the food had to be obtained by the mice. The jar was fitted with a metal screw top with an opening of 1 inch in diameter, which prevented the removal of the disc by the mice.

Male albino mice supplied by the Rockland Farms were used in this study. They were 21 days old and weighed from 7-9 gm. when placed on the experimental diets. They were divided into groups of eight and placed in cages with raised screen bottoms. The animals were weighed individually and as a group each week.

The basal ration described above did not support good growth in our mice (table 1). The weights reached a plateau at the end of the fourth week on this diet and many of the typical symptoms of a mild biotin deficiency were noted in the animals. The rough fur coat and

TABLE 1  
*Growth response.*

RATION	BODY WEIGHT (AVERAGE OF 8 MICE) AFTER			
	2 Weeks	4 Weeks	6 Weeks	8 Weeks
Basal	20	20	20	23
Basal + Biotin	20	24.5	23	25
Basal + Folic acid	21	25.5	26	28.5
Basal + Biotin + Folic acid	21	27	29	32
Basal + Sulfasuxidine	16	14	Dead	..
Basal + Sulfasuxidine + Biotin	17	19	16.5	Dead
Basal + Sulfasuxidine + Folic acid	18	21	22	20

curvature of the spine are shown in picture A which was taken after the mice had been on this diet  $4\frac{1}{2}$  weeks. In another experiment biotin was added to the basal diet at a level of 8  $\mu$ g. per 100 gm. of ration. The general appearance of the animals was much improved and the animals grew better. A mouse which received the basal ration plus biotin is shown in picture B.

A superfiltrol eluate (eluate factor or folic acid concentrate) was prepared according to the procedure of Hutchings et al. ('41). This concentrate was added to the basal ration at the equivalent of 4 gm. of solubilized liver per 100 gm. of ration. A group of mice receiving this supplement grew better than the animals receiving the basal ration (table 1). The difference in weight indicates that the mouse requires a source of folic acid when maintained on a completely artificial diet.

Another group of mice was given the basal diet to which had been added 8  $\mu$ g. biotin and a folic acid concentrate the equivalent of 4 gm.

of solubilized liver per 100 gm. of ration. The general appearance of the mice was very good as is seen in picture C, which was taken after the mice had been on this diet 4½ weeks. This group of mice showed greater gains in weight than the groups receiving either supplement alone as we were dealing with a complicated deficiency in the previous experiments. The animals were kept on this ration for 17 weeks and appeared normal in all respects. The individual weight of the animals at this time varied from 34–37 gm.

Since it was believed that mice can synthesize small amounts of biotin and folic acid, sulfasuxidine was incorporated in the basal diet to the extent of 0.6 gm. per 100 gm. of ration. A group of mice receiving this modified diet began to lose weight after the third week and all the animals were dead at the end of 6 weeks. An uncomplicated biotin deficiency was produced by supplementing this ration containing the drug with a folic acid concentrate. This diet supplemented with folic acid gave better growth responses but a typical alopecia developed after 4 weeks. The alopecia was well defined and is seen in picture D. It has been possible to cure and prevent this alopecia with biotin and definite growth of hair was seen after 2 weeks treatment. In another series of mice a severe folic acid deficiency was produced by feeding the basal diet containing the drug together with biotin at 8 µg. per 100 gm. of ration. No alopecia was seen in these animals although the hair coat was untidy. The administration of a folic acid concentrate restored the mice to apparent normal nutrition.

#### DISCUSSION

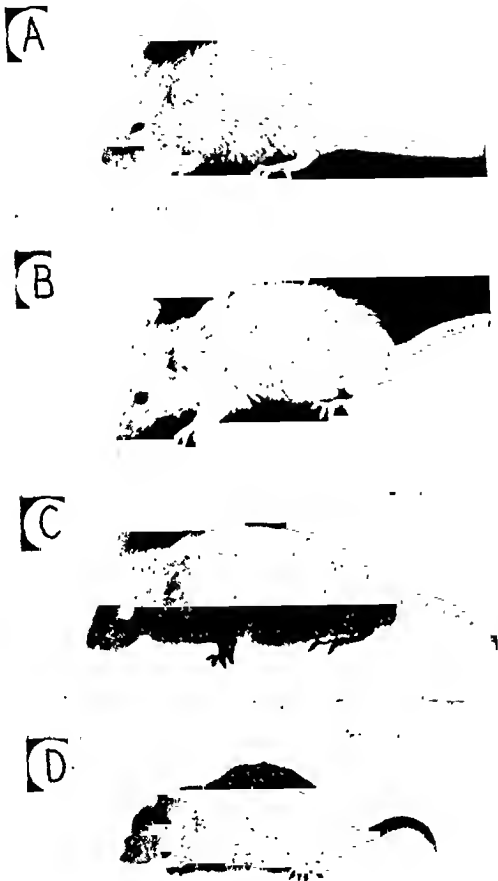
The supplementation of the basal ration with biotin and a folic acid concentrate gave a definite growth response. While rats maintained on a similar basal ration can synthesize adequate amounts of biotin and folic acid, it would appear that the bacteria in the intestines of the mouse are unable to meet the full requirements for this species. Since only a mild biotin and folic acid deficiency was produced on the basal ration it is believed that small amounts of these vitamins were synthesized in the intestinal tract but not in adequate amounts for normal growth. A severe biotin deficiency in our animals resulted in a well defined alopecia. A similar alopecia which responds to inositol has been described by Woolley ('40 a, b). Additional work is now in progress to ascertain if biotin is involved in the utilization or absorption of inositol or both.

## SUMMARY

1. Biotin and folic acid are required by mice maintained on a completely artificial diet.
2. A severe biotin deficiency in mice is characterized by alopecia.
3. Biotin and folic acid deficiencies produced in mice on a synthetic ration are more acute when 0.6% sulfasuxidine is included in this ration.

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- A A mouse which received the basal ration.  
B A mouse which received the basal ration plus biotin.  
C A mouse which received the basal ration plus biotin and a folic acid concentrate.  
D A severe biotin deficiency in a mouse characterized by a well defined alopecia.



# DIGESTIBILITY AND BIOLOGICAL VALUE OF SOYBEAN PROTEIN IN WHOLE SOYBEANS, SOYBEAN FLOUR, AND SOYBEAN MILK<sup>1,2</sup>

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The production of soybeans in this country has greatly increased within recent years. While only 29,000,000 bushels were produced in 1936, the yearly crop had increased to nearly 80,000,000 bushels by 1940, and the goal for 1943 was 216,000,000 bushels (Gortner and Gunderson, '44). Although the bulk of the domestic crop is not destined for human consumption, an important though minor fraction makes its contribution to the human dietary. It has been reported in this connection that 35,000,000 bushels of the 1943 crop would be available in the form of low-fat or high-fat soya flour for consumption by our own populace and by recipients of Lend-Lease (Gortner and Gunderson, '44).

Soybeans are exceptionally rich in protein<sup>3</sup> and it is of considerable practical importance to have as much information as possible on the nutritive qualities of this constituent of the bean. Provided it is of satisfactory nutritional value, the protein in soya products could afford a comparatively inexpensive supplement to the more common sources of food protein.

Experiments with animals have indicated that the protein of whole soybeans, after an appropriate heat treatment, possesses good nutritional qualities and is superior to the proteins of other legumes in its growth-promoting properties (Everson and Heckert, '44). Animal experimentation has also uncovered one of the limitations of the nutritive quality of soybean protein for it has been found that the addition of cystine or methionine improves the utilization by rats and chicks of the protein of both raw and cooked beans (Hayward and

<sup>1</sup>Supported by a grant from the Nutrition Foundation, Inc

<sup>2</sup>Presented at the meeting of the Michigan Academy of Science, Arts and Letters, Ann Arbor, March 17, 1944

<sup>3</sup>The beans used in the present investigation contained about 34% on the basis of N  $\times$  5.71.



Hafner, '41). Although these studies with animals are invaluable and have a bearing on human as well as on animal nutrition, experiments with human beings are also desirable. Research of this type is rather costly and requires the painstaking cooperation of a number of individuals. The results of such investigations, however, have the advantage over those obtained in animal studies of being more directly applicable to problems in human nutrition. There is a scarcity of reports of studies with human subjects on the nutritive value of the protein of soybeans. Most of the recorded investigations have dealt with the growth-promoting qualities of milk substitutes containing soybean protein for infants. Only one study on the biological value of soya protein appears to have been carried out with adult human subjects. Cheng, Li, and Lan ('41) reported that the biological value of the protein in soybean curd (protein content of diet 2.6%) was 64%. This value was based on data secured from one 3-day collection period on three subjects for both the experimental soya curd diet and a low-nitrogen basal diet.

The present research was undertaken to study the digestibility and the biological value of soybean protein in adult human subjects. The products examined were whole soybeans<sup>4</sup>, soyflour<sup>5</sup>, and soymilk.<sup>6</sup>

#### EXPERIMENTAL

The method employed was that used by Murlin and his associates ('41) to obtain comparable information on the protein in different breads. This procedure involves a comparison of the amount of urinary and fecal nitrogen excreted by subjects fed an especially designed diet containing egg protein, with the amount of nitrogen excreted by the same individuals when fed a similar diet containing the protein to be tested in place of the egg protein. The diet was planned to supply approximately four-fifths of the food nitrogen from either whole egg or the product under investigation, in adjacent test periods. Approximately one-half of the remaining dietary nitrogen was contained in cream, and the rest in the form of fruit, vegetables, and accessories. The total protein level of the diet was maintained at or near 5%

<sup>4</sup> Run-of-the-mill field-grown soybeans were used rather than a single variety. Studies with purified soybean protein from five varieties have shown little, if any, difference in the utilization between the varieties by the rat (G. Everson, personal communication). The soybeans were obtained through the courtesy of Dr. Percy Julian of the Glidden Company, Chicago.

<sup>5</sup> Staley's Soyflour no. 2 was used. It was secured through the courtesy of the A. E. Staley Manufacturing Company, Peoria.

<sup>6</sup> The soybean milk, "Mull-Soy," was generously provided by The Borden Company.

of the total calorie intake and the ratio of carbohydrate to fat was kept constant. Two of the diets eaten in the present study by one of the experimental subjects (F. G.) are shown in table 1. In calculating the biological value of the protein under study, Murlin and his associates

TABLE 1  
*Sample diets (F.G., diet squad II).*

STANDARD EGG DIET — PERIOD II					WHOLE SOYBEAN DIET — PERIOD III				
Foodstuff		Calories			Foodstuff		Calories		
		Prot.	Carbo.	Fat			Prot.	Carbo.	Fat
	gm.					gm.			
Egg <sup>1</sup>	246	130.2	..	241	Cooked				
Cream	160	12.7	25.4	535	soybeans 94	131.8 <sup>2</sup>	45.6 <sup>2</sup>	184	
Starch					Cream 160	12.7	25.4	535	
cracker	2X <sup>3</sup>	2.3	451.2	124	Starch				
Lettuce	60	2.0	5.7	1.2	cracker 2X <sup>3</sup>	2.3	451.2	124	
Salad					Lettuce 60	2.0	5.7	1.2	
dressing	31	..	..	284	Salad				
Vitamin					dressing 37	..	..	342	
suppl.	0.25	0.3	..	..	Vitamin				
Orange					suppl. 0.25	0.3	..	..	
juice 150		4.1	65.0	2.8	Orange				
Margarine 40		1.2	..	305	juice 150	4.1	65.0	2.8	
Dextri-					Margarine 40	1.2	..	305	
maltose 172		3.1	670	..	Dextri-				
Apple 300		0.2	171	11.1	maltose 172	3.1	670.0	..	
Sucrose 22		..	128	..	Apple 300	0.2	171	11.1	
		156.1	1516.3	1504.1	Sucrose 20	..	80.0	..	
						157.7	1513.9	1505.1	
Total calories, 3176.7					Total calories, 3176.7				
Protein, 4.91%					Protein, 4.96%				
Carbohydrate, 47.7%					Carbohydrate, 47.7%				
Fat 47.3%					Fat 47.3%				

<sup>1</sup> Eaten as whole egg omelet.

<sup>2</sup> The percentage of protein in soybeans for use in the calculation of the protein calories was obtained by multiplying the percentage of soybean nitrogen by the factor 5.71, which is the nitrogen factor given by Jones ('31).

<sup>3</sup> The soybean carbohydrate calories were based on the percentage of available carbohydrate. Adolph and Kao ('33) have reported that 40% of soybean carbohydrate is utilized by the animal body.

<sup>4</sup> The starch cracker was eaten to supply calories with little nitrogen. It was patterned after the cracker employed by Sumner and Murlin ('38) in one of their studies. The batter for the cracker used in the present investigation consisted of corn starch, 600 gm., gum acacia 32 gm., Crisco 80 gm., alum baking powder 20 gm., Karo 80 gm., salt 8 gm., and water 800 gm. 270 gm. of this batter poured into two pie tins and baked slowly for 1 hour provided two starch crackers. Two such crackers were eaten by each subject.

('41) made the tacit assumption that the egg protein was completely utilized. The calculated biological value was based, therefore, on egg protein as 100.

The experimental subjects were all males from the Medical School staff and student body. Owing to the nature of the research, only a limited number of individuals could be studied together. Experiments were carried out with three separate diet squads, one squad being studied at a time. Data on the ages, heights, body weights, and the distribution of calories in the experimental diets for each subject in the different diet squads, together with dates showing the duration of the experiments with each squad, are given in table 2.

The subjects ate all of their meals in one of the departmental laboratories which served both as a dining room and kitchen. During a preliminary interval the individuals were fed a standard egg diet for a period of several days (6 to 10 days), during which time adjustments were made in the diet and the subjects became familiar with the routine of eating together and of collecting feces and urine. After the preliminary interval, the first soybean product period began. This was followed by egg and soya product periods, so spaced that a soya product period was always adjacent to an egg period. All of the experimental periods were 6 days in length.

The subjects remained in good health throughout the study and the data in table 2 show that changes in body weight were only moderate. The diet promoted normal gastrointestinal function in all of the subjects. More frequent defecation and better-formed stools were observed during each of the soybean product periods than during the standard egg diet periods. The high satiety value of the ingested soybean products was noted.

The soybean products employed as the source of the bulk of the nitrogen in the different experimental diets were prepared for consumption as described below. The whole soybeans were autoclaved with 2 parts of water at 15 pounds pressure for 1 hour. This treatment rendered them comparable in softness and flavor to soybeans cooked for several hours at normal atmospheric pressure. They were reheated for serving. The soyflour was mixed with water and salt ( $2\frac{1}{2}$  oz. flour,  $\frac{3}{4}$  cup water, and  $\frac{1}{4}$  teaspoon salt) and autoclaved for 1 hour. Individual portions of the soya products were cooked daily. Although the soymilk was not autoclaved, heat had been employed in the course of its manufacture. The soymilk was warmed before drinking. Whole egg was used in the form of an omelet. The eggs, secured from one local source, did not vary appreciably in protein con-

TABLE 2

*The diet squads\* and the caloric distribution in the experimental diets.*

Date	SQUAD	AGE	HEIGHT	BEGINNING WEIGHT	WEIGHT CHANGE	CALORIES PER DIEM			
						Total intake	Distribution		
							Prot.	Carbo.	Fat
		yrs.	cm.	kg.	kg.		%	%	%
Nov. 16 — Dec. 22, '42	I W. C.	31	178.0	67.6	- 1.8	2607 <sup>b</sup> 2594 <sup>c</sup> 2605 <sup>d</sup>	5.0 4.7 4.9	47.5 47.6 47.5	47.5 47.7 47.6
	S. K.	24	180.2	75.5	+ 0.5	3607 <sup>b</sup> 3593 <sup>c</sup> 3607 <sup>d</sup>	5.0 4.7 5.0	47.5 47.6 47.4	47.5 47.7 47.6
	K. P.	22	190.6	67.0	+ 0.7	3607 <sup>b</sup> 3593 <sup>c</sup> 3607 <sup>d</sup>	5.0 4.7 5.0	47.5 47.6 47.4	47.5 47.7 47.6
	R. R.	22	171.2	62.5	- 0.7	2607 <sup>b</sup> 2591 <sup>c</sup> 2605 <sup>d</sup>	5.0 4.7 4.9	47.5 47.6 47.5	47.5 47.7 47.6
	II R. C.	22	184.2	86.7	- 0.2	3799 <sup>b</sup> 3805 <sup>c</sup> 3804 <sup>d</sup>	4.9 4.0 4.9	47.6 47.5 47.6	47.5 47.6 47.5
	J. G.	23	165.1	64.5	0.0	2995 <sup>b</sup> 2987 <sup>c</sup>	5.1 5.1	47.6 47.5	47.4 47.4
	F. G.	21	178.0	61.0	+ 0.1	3177 <sup>b</sup> 3177 <sup>c</sup> 3179 <sup>d</sup>	4.0 5.0 5.0	47.7 47.5 47.4	47.3 47.3 47.6
	J. H.	22	170.9	67.5	+ 0.5	3208 <sup>b</sup> 3206 <sup>c</sup> 3208 <sup>d</sup>	4.9 5.0 4.9	47.7 47.5 47.5	47.4 47.5 47.6
	G. V.	24	174.9	90.4	- 2.2	3167 <sup>b</sup> 3168 <sup>c</sup> 3168 <sup>d</sup>	4.0 4.9 4.8	47.6 47.6 47.7	47.5 47.5 47.5
	J. Y.	22	183.5	74.0	+ 0.5	3500 <sup>b</sup> 3504 <sup>c</sup> 3503 <sup>d</sup>	5.0 5.0 5.0	47.5 47.5 47.5	47.5 47.5 47.5
	III R. C.	22	184.2	86.3	0.0	3798 <sup>b</sup> 3797 <sup>c</sup>	4.9 4.8	47.6 47.6	47.5 47.6
	B. C.	22	181.4	77.5	- 0.5	3584 <sup>b</sup> 3597 <sup>c</sup>	4.0 5.0	47.6 47.5	47.5 47.5
Feb. 2 — Mar. 16, '43	A. F.	22	180.0	71.4	- 1.6	3302 <sup>b</sup> 3299 <sup>c</sup>	5.0 4.9	47.6 47.6	47.4 47.5
	F. G.	21	178.0	62.1	0.0	3190 <sup>b</sup> 3190 <sup>c</sup>	4.9 4.9	47.6 47.6	47.5 47.5
	J. H.	22	170.9	68.7	+ 0.5	3207 <sup>b</sup> 3209 <sup>c</sup>	4.9 4.9	47.6 47.6	47.5 47.5
	S. Z.	23	169.0	63.1	+ 1.6	2989 <sup>b</sup> 2985 <sup>c</sup>	5.1 5.0	47.5 47.5	47.4 47.5
May 24 — June 22, '43									

\* Three of the subjects served on two diet squads; all others served only once.

<sup>b</sup> Standard egg diet containing whole egg omelet as the chief source of protein.<sup>c</sup> Diet containing autoclaved whole soybeans as the chief source of protein.<sup>d</sup> Diet containing soybean "milk" as the chief source of protein.<sup>e</sup> Diet containing autoclaved soybean flour as the chief source of protein.

tent as determined by nitrogen analyses. One brand of oleomargarine was employed throughout the study; it was much lower in nitrogen than butter. The cream (36%) was secured by special arrangement from a local dairy. The orange juice was prepared from California Valencias or Florida oranges, depending on the season; the apples were largely Northern Spies. Dextrimaltose<sup>7</sup> and sucrose were used to augment the carbohydrate calories. Salad dressing was always made from the same brands of commercial salad oil and distilled vinegar. One vitamin capsule<sup>8</sup> daily provided 1.5 mg. of thiamine chloride, 2 mg. of riboflavin, 1 mg. of calcium pantothenate, 10 mg. of niacin, 250 µg. of pyridoxin, and yeast and liver extract. The gelatin shell of the capsule was not consumed with the vitamins.

All analyses for nitrogen were made by the macro Kjeldahl method. Foods were sampled periodically and nitrogen determinations made on aliquots of pooled samples. Urine was analyzed daily for the last 4 days of each period. Nitrogen analyses were made on the fecal material collected during each 6-day period. Charcoal markers taken with the first meal of each period permitted separation of the feces of one period from those of another. The fresh feces were kept covered with ethanol containing 1% sulfuric acid until dried on the steam cone. Nitrogen determinations were made on aliquots of the ground dried material.

For purposes of calculation of the biological value, the average daily nitrogen excretion in the feces and in the urine by subjects fed a soybean product diet was compared to that of the same subjects fed the comparable standard egg diet, according to the method of Murlin and his associates ('41), details of which are shown in table 3. In most cases the daily averages were obtained from the data for two periods for each type of diet. In the experiment with diet squad II, for instance, diets were eaten in the following order: preliminary egg, whole soybean, standard egg, whole soybean, soymilk, standard egg, soymilk. The daily average nitrogen excretion during the two whole soybean periods was compared in the prescribed manner with that of the two standard egg periods; the daily average nitrogen excretion during the two soymilk periods was similarly compared. The urinary excretion of nitrogen during the two standard egg periods was not identical—sometimes a few decigrams less nitrogen was excreted in the second period by a given individual than in the first, sometimes

<sup>7</sup> We are indebted to Mead Johnson and Company for the Dextrimaltose no. 1 used in these experiments.

<sup>8</sup> Kindly provided by the Gelatin Products Company.



vice versa. In some instances data were available for certain subjects for only one period in which a certain type of diet was eaten. Thus, in the first diet squad experiment four individuals ate whole soybeans for one period instead of the usual two and the daily averages for this one food product were based on one period for these subjects. Similarly, the subjects of diet squad III ate the standard egg diet for only one period owing to the untimely interruption of the experiment by the Detroit race riot.

#### DISCUSSION OF RESULTS

The values for the true digestibility and for the biological value of soya products tested are given in table 3 for each of the experimental subjects. The data in table 3 show little variation in the average protein digestibility between the soybean products used in this study. The average values for autoclaved whole soybeans, autoclaved soyflour, and soymilk are 90.5, 94.0, and 89.6%, respectively. For purposes of comparison it may be mentioned that Tso and Chu ('31), in studies with infants, recorded values averaging 80% when soya protein was fed in the form of soybean "milk."

The average biological values for maintenance for the three soya products are not widely dissimilar, being 94.5% for the protein in cooked whole soybeans, 91.7% for cooked soybean flour, and 95.3% for soybean "milk." It is noteworthy for purposes of comparison that the biological values for maintenance of the protein of a wheat cereal biscuit, a whole wheat bread, and a white bread as determined by the method employed in the present study were 81.6%, 77.8%, and 74.6%, respectively (Murlin, Marshall, and Kochakian, '41).

The comparatively high biological value of soybean protein as determined in our studies with human subjects is in accord with the the results of investigations with animals which have given evidence of the good nutritional qualities of the protein of this legume. Not only is suitably prepared soybean protein capable, as the sole source of protein in the diet, of supporting good growth in experimental animals, but soybean flour supplements greatly improve the nutritive value of patent flour (Jones and Devine, '42).

It is well known that the biological value of dried uncooked soybean protein may be improved by a certain degree of heating (Johnson, Parsons, and Steenbock, '39). No attempt was made in the present study to assess the effect of heat on the biological value of protein in the soybean products eaten. The whole soybeans and the soybean

flour used in our experiments were cooked (autoclaved) for the practical purpose of making them palatable.

The nitrogen balances calculated for the different experimental periods indicated that the level of protein in the experimental diets was well-suited to bring out differences between the egg protein and the protein of the various soybean products studied. The average daily nitrogen balance for all of the egg diet periods was  $+0.787$  gm., while the comparable balance values for the whole soybean, soyflour, and soymilk diets were  $-0.147$ ,  $+0.298$ , and  $-0.162$  gm., respectively. As shown in table 3, the subjects excreted more nitrogen in the feces when fed the soya product diets than when fed the standard egg diet. This finding was in part responsible for the less favorable nitrogen balances observed when soybean diets were consumed. Rose and MacLeod ('25) observed that the "cost of digestion," as assessed by the excretion of fecal nitrogen, was greater for soybean curd than for the protein in meat, and in bread and milk.

Both the digestibility and the biological value of a protein are important, of course, in determining its over-all nutritive value. By multiplying the digestibility by the biological value a quantitative expression is obtained which might be termed the nutritive index. The comparative nutritive index of the protein in whole cooked soybeans, as determined in the present maintenance experiments, is 0.9 multiplied by 94.5%, or 85 compared to egg taken as 100.\* Although the exigencies of digestion and consequent loss of nitrogen in the feces reduce somewhat the gross nutritive value of the soya protein, the soybean appears to be a valuable adjunct to the more common sources of dietary protein.

#### SUMMARY

The average true digestibility in adult human subjects of the protein in cooked whole soybeans, in cooked soybean flour, and in soybean milk was found to be 90.5%, 94.0%, and 89.6%, respectively.

The average biological value of soybean protein for maintenance in adult human subjects, as determined by the method of Murlin et al. ('41) in which the protein of whole egg is employed as a standard, was found to be 94.5% for the protein in cooked whole soybeans, 91.7% for that in cooked soyflour, and 95.3% for that in a commercial soybean "milk."

\*A value of 100 would presuppose not only complete digestibility of egg protein but also a biological value of 100%. Actually, the average biological value of the protein of whole egg, as determined in experiments with young rats (Mitchell and Carmen, '26) is 94%. The true nutritive index of whole egg protein, as well as that of soybean protein, is, therefore, somewhat lower than the value given in the text.



Under the conditions of the present study less favorable nitrogen balances were observed when the experimental subjects were fed diets containing soybean products than when they were fed a standard egg diet, owing largely to greater loss of soybean nitrogen in the feces.

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# THE CHOLINE AND PYRIDOXINE CONTENT OF MEATS<sup>1</sup>

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(Received for publication June 1, 1944)

Although choline and pyridoxine are recognized as essential factors for normal animal metabolism there is a paucity of information concerning the distribution of these substances even in the main dietary constituents. Since chemical and microbiological methods for the determination of choline (Engel, '42) and pyridoxine (Atkin et al., '43) were available, we have used these methods to determine the choline and pyridoxine content of a number of fresh, cooked and commercially prepared meats.

## EXPERIMENTAL

The methods of preparing the samples have been reported previously (McIntire et al., '43), (Schweigert et al., '44) and (McIntire et al., '44). All determinations were made on undried fresh and cooked meats. Values reported on the dry basis were calculated from results of the determinations on fresh meats.

The method used for the determination of choline was essentially that reported by Engel ('42). Samples of fresh and cooked meats were minced finely and extracted over night with boiling methanol. The alcohol extracts were evaporated to dryness on a steam bath, and the residues were saponified for 2 hours with boiling aqueous barium hydroxide. After saponification the barium soaps were filtered off, and the choline was precipitated as choline reineckate by adding 5 ml. of an alcoholic solution of 2% ammonium reineckate to the filtrate. Complete precipitation of the choline reineckate was assured by allowing the mixture to stand in a  $-4^{\circ}\text{C}.$  cold room for 12 hours. The bright red choline reineckate precipitate was then washed with 2-5 ml. portions of cold ethanol and water until the washings were free of color. The choline reineckate was dissolved in approximately 15 ml. of acetone and the concentration determined by measuring the density

<sup>1</sup> Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

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of the color in an Evelyn colorimeter. This method proved very satisfactory and duplicate determinations with few exceptions checked within 5%.

A modification of the yeast method of Atkin et al. ('43) was used for the determination of pyridoxine. Yeast cultures were grown in 125 ml. Erlenmeyer flasks in a 30°C. incubator without shaking for 19 hours. Maximum liberation of the pyridoxine was attained when the meat samples were autoclaved for 1 hour at 20 lbs. pressure in 180 ml. of 0.1 N HCl. Recoveries varying from 83 to 101% were obtained under these conditions. Considerable variation was observed in duplicate analyses although all values recorded in this paper were checked within 10%. The choline and pyridoxine contents of veal, lamb, pork, and beef as well as several special organs and commercially prepared meats were determined (table 1).

#### DISCUSSION

Choline values for the various meats agree with the available values reported by Engel ('43), who has made a general survey of the choline content of animal and plant products. Our values for roast veal were 133 to 144 mg., for lamb chops 76 mg., and for ham 101 to 129 mg. of choline per hundred grams of meat compared to 113 mg. for veal roast, 106 mg. for lamb chops, and 88 mg. per hundred grams for ham reported by Engel. Our choline values for kidney and liver, 240 to 280 mg. and 470 to 570 mg. per hundred grams compare with 330 and 486 to 708 mg. of choline per hundred grams of kidney and liver reported by Engel.

Most pyridoxine values obtained in this work are in good agreement with those obtained by Henderson et al. ('41) with the rat assay method, although some of our values for the muscle meats are slightly lower. These discrepancies are attributed to the fact that fat was trimmed from the meats assayed by Henderson et al. They reported 0.3 mg. of pyridoxine for lamb muscle, 0.59 mg. for ham, and 0.4 mg. for veal per hundred grams. The range of our values for these meats are 0.22 to 0.33 mg. for lamb, 0.25 to 0.37 mg. for ham and 0.25 to 0.45 mg./100 gm. for veal. The values for organ meats are in good agreement with the values reported by Henderson et al. ('41) which are 0.44 mg. of pyridoxine for beef kidney, 0.24 mg. for beef heart, 0.73 mg. for beef liver and 0.12 mg./100 gm. for beef tongue. Our corresponding values for these meats were 0.36 to 0.42 mg. for beef kidney, 0.29 mg. for veal heart, 0.5 to 0.99 mg. for beef liver and 0.13 mg./100 gm. for beef tongue.

Vitamin retention studies were carried out after cooking veal and lamb and curing ham. The method for calculating retentions has been previously described (McIntire et al. '43a). The veal samples were roasted, braised and stewed and the lamb samples were roasted, broiled, and stewed.

The destruction or leaching of choline from the meats during any of the cooking or curing processes is negligible since 87 to 114% of the choline was retained in the meat.

TABLE 1  
*Choline and pyridoxine content of meats. (mg./100 gm.)*

SAMPLE	CHOLINE				PYRIDOXINE			
	Fresh		Dry		Fresh		Dry	
	Range	Avg	Range	Avg	Range	Avg.	Range	Avg.
<b>Veal</b>								
Leg	95-108	102	366-432	389	.34-.41	0.37	1.3-1.6	1.4
Roast leg	125-141	132	338-392	360	.20-.21	0.20	.50-.57	.53
Shoulder	83-100	93	268-373	337	.25-.38	.30	.81-1.4	1.1
Roast shoulder	133-143	139	310-376	343	.12-.15	.14	.30-.37	.33
Sirloin chop	87-105	96	242-404	317	.36-.45	.41	1.0-1.7	1.4
Braised chop	128-157	140	242-342	285	.10-.12	.11	.21-.24	.23
Shoulder chop	92-101	97	307-422	376	.32-.38	.35	1.1-1.4	1.2
Braised chop	149-156	154	317-400	366	.11-.14	.12	.22-.34	.28
Stew meat	94-100	96	336-400	367	.32-.34	.33	1.3	1.3
Cooked stew	137-149	142	360-378	370	.09-.10	.10	.24-.27	.26
<b>Lamb</b>								
Leg	75-92	84	262-317	290	.24-.33	.29	.80-1.1	.93
Roast	122-124	123	284-295	290	.10-.13	.12	.23-.31	.27
Sirloin chop	75-77	76	179-198	189	.21-.22	.22	.50-.56	.53
Broiled chop	100-126	113	204-252	228	.08-.13	.11	.17-.26	.22
Stew meat	76-82	79	222-230	226	.22-.23	.23	.62-.66	.64
Cooked stew	116-128	122	247-291	269	.05-.06	.06	.11-.14	.13
<b>Pork</b>								
Ham	101-129	120			.25-.37	.33		
Cured ham	98-129	122			.15-.21	.19		
<b>Beef</b>								
Liver	470-570	510			.50-.99	.71		
Round	65-70	68			.31-.43	.37		
Tongue	108	108			.13	.13		
Heart	170	170			.29	.29		
Braised heart	200-275	238						
Kidney	240-284	262			.36-.42	.39		
Brain	399-420	410			.16	.16		
<b>Miscellaneous</b>								
Bologna	60	60						
Frankfurters	57	57			.13	.13		
Pork links	48	48						
Canadian bacon	80	80						



A low pyridoxine retention in meat after cooking was quite surprising in view of the fact that this vitamin is considered to be one of the heat stable members of the B complex. Retentions of pyridoxine in the meat after various cooking methods ranged from 14 to 42% and retention in ham after curing averaged 57%. A few of the drippings from the cooked meat were available for analysis, but all were found to contain less than 6 % of the total vitamin in the meat. A summary of the pyridoxine retention studies is shown in table 2.

TABLE 2  
*Retention of pyridoxine in meat. (All values in %.)*

VEAL			LAMB			HAM
Roasting	Braising	Stewing	Roasting	Broiling	Stewing	Curing
41	19	18	28	40	18	53
36	22	18	29	28	14	59
42	17					58
26	22					58
31	15					
29	20					
Avg. 34	19	18	28.5	34	16	57

A few correlations between the type of cooking and the pyridoxine retention are apparent. Roasting and broiling resulted in higher retention than stewing and braising. The average retention after roasting veal was 34% and after roasting lamb 28%. After broiling lamb the average amount of pyridoxine retained was 34%. The retention after stewing veal and lamb was 18 and 16%, respectively, while retention after braising veal was 18%.

Similar correlations were found for thiamine retention in veal and lamb after these cooking processes (McIntire et al., '43). It was suggested at that time that the extracting action on the meat of the condensing vapors and surrounding liquid during the braising and stewing processes was responsible for the greater thiamine losses from the meat. The pyridoxine retentions after these cooking processes can also be explained on this basis.

#### SUMMARY

A survey of the choline and pyridoxine content of veal, lamb, pork, beef, special organ meats, and commercially prepared meats has been made. Muscle meats contain from 0.22 to 0.45 mg. of pyridoxine and 70 to 144 mg. of choline per hundred grams. Kidney, heart and liver are somewhat higher.

The vitamin retention after curing and cooking was studied. Between 87 and 114% choline retention was observed while the pyridoxine retention ranged from 14 to 42% after cooking. An average of 57% of the pyridoxine was retained in ham after curing.

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# INADEQUACY OF LACTOSE AND BETA-LACTOSE AS DIETARY CARBOHYDRATES FOR THE RAT

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A strain difference in the development of cataract has been reported in the rat both in relation to the time of onset and to the degree of incidence following lactose feeding (Mitchell, '36). Further studies have demonstrated that the cataractogenic properties of these rations were not due to lactose per se but rather to galactose liberated from this sugar on hydrolysis (Mitchell, '35; Day, '36). In the present communication we wish to report the inadequacy of lactose and beta-lactose<sup>1</sup> as the sole dietary carbohydrate in purified rations for the rat, an inadequacy varying both in extent and degree with the strain and not shared by galactose, glucose, sucrose or corn starch when one of the latter replaces lactose or beta-lactose in the ration.

## PROCEDURE AND RESULTS

Two strains of rats were employed in the present experiment: (1) animals of the Long-Evans strain (both colored and albino); and (2) animals of the U.S.C. strain<sup>2</sup> (albino). At weaning (21-23 days) litter mates of both sexes were placed on eight different dietary regimes (table 1). Seven groups of rats were maintained on diets differing solely in composition of dietary carbohydrate and an eighth group (H) was maintained on Purina dog chow which was supplemented once a week with lettuce. A total of ninety-four Long-Evans and sixty-four U.S.C. rats were employed in the following experiment.

After 72 hours on the feeding regimes, Long-Evans rats on the beta-lactose ration differed markedly from litter mates on control diets or animals of the U.S.C. strain on the same diet. Ingestion of the beta-lactose ration resulted in a syndrome consisting of severe diarrhea,

<sup>1</sup> Beta-lactose or anhydrous lactose is formed by crystallizing lactose at a temperature of 93.5°C. It is a white crystalline powder, sweeter than lactose, and about twice as soluble as the latter in water. In the presence of water at 75°C. it gradually reverts to ordinary lactose.

<sup>2</sup> A modified Wistar strain.

able from litter mates on control rations, with the exception of retarded growth. Subsequent to this period, symptoms similar to those in the Long-Evans strain developed. In general, alopecia was not as marked as in the latter strain; only  $\frac{1}{4}$  of the rats exhibited thinning or loss of fur with the affected area primarily the lower back and abdomen. At autopsy the only gross abnormality observed was a marked dilation of the cecum<sup>3</sup> approaching twenty times its extent in litter mate controls. No significant difference was observed between the two strains after feeding the other diets.

#### *Effects of previous diet on beta-lactose feeding*

Ten Long-Evans rats were placed at weaning on rations E and H (five animals per group). Both diets were fed ad libitum. After 28 days of feeding rats were transferred to a beta-lactose ration (diet B). No significant difference in response was noted between the two groups. Survival averaged  $14 \pm 3$  days. By the seventh day alopecia was marked in both series, animals developed acute diarrhea, lost weight rapidly, and at autopsy averaged 30% less than when placed on the beta-lactose ration. There is some evidence that the age at which rats were started on the beta-lactose ration affected response to feeding. When rats were placed on a beta-lactose ration at weaning, survival averaged 4.9 days; when placed on the ration at 49 days of age, survival averaged 14 days; when started at 150 days of age (three rats) animals remained free of symptoms for 28 days at which time feeding was discontinued.

#### *Effects of previous maternal diet on lactose and beta-lactose feeding of young*

The experimental animals of the following series consisted of young born to mothers of the U.S.C. strain that (1) had been raised from weaning on a stock diet and bred on the same ration (Deuel et al., '33), (2) raised from weaning on a stock diet but changed to a Sherman diet 2 months before mating (I), and (3) raised from weaning and bred on a Sherman diet for nine generations (II). At weaning the young were placed on rations A and B which were fed ad libitum.

No significant difference was observed at autopsy in animals dying on lactose or beta-lactose rations regardless of the previous diet of the parents. With the exception of young born on a stock diet, how-

<sup>3</sup> We wish to express our sincere appreciation to Professor E. M. Hall, Department of Pathology, University of Southern California Medical School, for his help at autopsy.

ever, a certain percentage of rats placed on lactose or beta-lactose rations survived 8 weeks of feeding at which time the feeding was discontinued. Subsequent to the third week a marked improvement occurred in those rats which did survive, the animals gained weight, new hair replaced the areas of alopecia, and diarrhea improved.

TABLE 2

*The effect of maternal diet on survival after transferring weaned rats to lactose or beta-lactose diets.*

PREVIOUS MATERNAL DIET	NUMBER OF ANIMALS	PERCENTAGE OF ANIMALS DYING	AVERAGE SURVIVAL TIME OF DECEDENTS <sup>1</sup>	PERCENTAGE OF ANIMALS SURVIVING
<b>Lactose tests (A)</b>				
Stock diet	12	100	25.8 ± 0.7	0
Sherman I	10	70	17.1 ± 1.8	30
Sherman II	10	60	24.7 ± 0.9	40
<b>Beta-lactose tests (B)</b>				
Stock diet	12	100	21.9 ± 0.9	0
Sherman I	10	80	16.1 ± 2.2	20
Sherman II	17	47	21.2 ± 1.0	53

<sup>1</sup> Including standard error of the mean calculated as follows:  $\sqrt{\frac{\sum d^2}{n}} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

Failure of survival on lactose or beta-lactose rations in rats born on a stock diet as compared with the control series appears to be significant, but the number of animals in each group is too small to permit a final statement on this matter. Of animals that failed to survive these rations no protective action on length of survival was noted that could be attributed to the previous maternal diet.

#### *Effect of dietary supplements on beta-lactose feeding*

The recent report by Bontwell et al. ('43, '44), that butter fat is superior to corn oil in promoting growth of rats maintained on purified rations containing lactose as the sole carbohydrate, suggested a possible interrelationship between the beta-lactose syndrome and dietary fat. Accordingly, corn oil was omitted from the ration and butter fat, oleomargarine, and lard incorporated to the extent of 10% of the diet replacing a similar quantity of beta-lactose. Groups of four rats each of the Long-Evans strain were placed on the above diets at weaning and fed the ration ad libitum. Animals receiving the lard supplement survived an average of 2.4 days, those receiving oleomargarine 3 days, and those on the butter fat supplement 3.2 days. Butter fat, oleo-

able from litter mates on control rations, with the exception of retarded growth. Subsequent to this period, symptoms similar to those in the Long-Evans strain developed. In general, alopecia was not as marked as in the latter strain; only  $\frac{1}{4}$  of the rats exhibited thinning or loss of fur with the affected area primarily the lower back and abdomen. At autopsy the only gross abnormality observed was a marked dilation of the cecum<sup>3</sup> approaching twenty times its extent in litter mate controls. No significant difference was observed between the two strains after feeding the other diets.

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No significant difference was observed at autopsy in animals dying on lactose or beta-lactose rations regardless of the previous diet of the parents. With the exception of young born on a stock diet, how-

<sup>3</sup> We wish to express our sincere appreciation to Professor E. M. Hall, Department of Pathology, University of Southern California Medical School, for his help at autopsy.

hydrate portion of the diet, the previous dietary history of the parents or such environmental conditions as temperature and humidity.

Whether strain differences alone could account for the above discrepancies is problematical although there is considerable evidence to indicate that rats of different strains may respond differently to the same dietary.<sup>10</sup> Long-Evans rats on a beta-lactose ration developed acute diarrhea within 24 hours of the initial feeding; U.S.C. rats on the same ration remained free of diarrhea for at least 8 days. Long-Evans rats on a beta-lactose ration survived an average of 4.9 days with 100% mortality; U.S.C. rats on the same ration survived 20 days with 28% showing spontaneous recovery and freedom from symptoms. Fifty per cent of the Long-Evans rats on a beta-lactose ration developed alopecia; U.S.C. rats on the same diet developed this condition in only half this number. Similar discrepancies were observed in respect to lactose feeding.

The pathologies observed on lactose and beta-lactose rations suggested the possibility of alterations in the intestinal flora acting as a contributory factor in these conditions. It has long been known that lactose feeding brings about changes in the pH of the contents of the whole intestinal tract below the duodenum (Hudson and Parr, '24; Robinson and Duneau, '31) and favors the development of an acidophilic flora (Rettger and Cbeplin, '23; Hudson and Parr, '24). Such changes have not been observed in diets containing glucose, sucrose, galactose or corn starch in place of the lactose. Inasmuch as microorganisms present in the intestinal tract are known to produce biotin, folic acid, inositol, and other members of the vitamin B complex in addition to vitamin K, the possibility of alterations of the intestinal flora from the normally proteolytic to the acidophilic type precipitating nutritional deficiencies either through failure of intestinal synthesis or through increased utilization of factors already present is worthy of further consideration. Boutwell et al. ('43) and Gyer et al. ('43) have suggested essentially the same possibilities. An analysis of earlier diets utilizing lactose or beta-lactose as the sole dietary carbohydrate has revealed the fact that in almost every case natural sources of the vitamin B complex were employed in addition to unextracted casein and fats such as lard or Crisco. The possibility of nutritional factors being present in these materials in sufficient concentration to make good any deficiency resulting from alterations in the intestinal flora has not been excluded.

<sup>10</sup> Experiments are now in progress with the Sprague-Dawley rat. Preliminary results indicate that the development of diarrhea in this strain on diets A and B is similar in extent and time of appearance to that observed in the U.S.C. strain.



The findings of Boutwell et al. ('43, '44) and Geyer et al. ('43) that butter fat and lard are superior to corn oil in promoting growth of rats receiving lactose as the sole carbohydrate would seem to indicate this very condition although Deuel et al. ('44) have noted no differences in nutritive values of butter fat and various vegetable fats when fed with skimmed milk powder. Geyer et al. ('43) have noted that rats maintained on a purified ration containing 48% lactose and 28% corn oil developed "rough and discolored fur coats, blood-stained noses and scaly paws (when the humidity was not abnormally high)." In their most recent report, Boutwell et al. ('44) have reported the occurrence of alopecia on a similar ration. Nielsen and Black ('44) employing diets containing 73% lactose and 5% corn oil reported kidney damage and many early deaths.

That alterations of the intestinal flora are not the sole or even the predominant factors involved in precipitating the "beta-lactose syndrome" might be indicated, however, by the failure of dietary supplements to modify the response to beta-lactose feeding in the Long-Evans rat. Butter fat, oleomargarine, lard, wheat germ oil, cream, biotin, and brewer's yeast were all ineffective in promoting survival or prolonging the appearance of symptoms. Of all the substances tested only liver gave some indication of a protective effect. Furthermore, the rapidity with which symptoms developed suggested that some factor other than altered intestinal synthesis is involved. Mitchell and co-workers ('39) have pointed out that the presence of the gluconate radical either as the sodium or calcium salt inhibits utilization of lactose by the rat. According to these workers the gluconate ion inhibited lactase activity in the intestinal tract resulting in definite toxic symptoms, retarded growth, poor survival, moderate to severe diarrhea and lowered blood sugar. In the present study data on blood sugar and lactase activity of the intestinal tract are lacking, but further studies on the effects of substances either of dietary or intestinal origin inhibiting lactase activity might provide information on the mechanism involved in the beta-lactose syndrome.

Variations in lactase activity might also be a factor in explaining strain differences. There is evidence to indicate that the diarrhea resulting from ingestion of lactose or beta-lactose is related to failure of enzymatic hydrolysis and the excess of lactose remaining as such in the alimentary tract. Failure of pectin and apple powder to counteract the diarrhea resulting from ingestion of beta-lactose rations would be consistent with this hypothesis. Inasmuch as the Long-Evans rat developed acute diarrhea within 24 hours after ingesting a beta-lactose

ration, contrasting with 8 and 10 days necessary for this condition to develop in the U.S.C. rat, differences in lactase activity of the two breeds might be expected. Further variations in lactase activity might be expected in U.S.C. rats exhibiting spontaneous recovery on a lactose or beta-lactose ration as contrasted with litter mates dying on the same diet.

#### SUMMARY

Male and female rats of the Long-Evans and U.S.C. strains were placed at weaning on purified rations containing lactose, beta-lactose, glucose, galactose, sucrose and corn starch as the sole source of carbohydrate. The following observations were made: (1) rats failed to survive on purified rations containing lactose or beta-lactose as the sole carbohydrate; (2) rats developed alopecia when fed the above lactose or beta-lactose rations; (3) a strain difference was observed in the incidence and degree of alopecia as well as the length of survival of rats fed lactose or beta-lactose rations; (4) rats fed a beta-lactose ration died sooner and developed more severe alopecia than those fed a lactose diet; (5) length of survival was correlated with severity of diarrhea; and (6) a relationship was observed between degree of mortality and previous maternal diet.

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## THIAMINE CONTENT OF HEN EGGS DURING INCUBATION<sup>1</sup>

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Relatively few studies have been made on the vitamin content of developing eggs. Suomalainen ('39 a) found that the vitamin A content decreased while vitamin C was synthesized (Suomalainen, '39 b). Snell and Quarles ('41) followed the concentration of several of the vitamins of the B complex in hen eggs during incubation. They reported that nicotinic acid and inositol were synthesized, but the total amount of pantothenic acid, riboflavin and biotin remained essentially unchanged. Woolley ('42) reported that the total content of inositol in the hen egg does not increase during incubation but the proportion of the total amount which is free and extractable by water does.

Williams, Taylor and Cheldelin ('41) have observed the B vitamin content of rat and chick organs at several different ages. Marked differences were found in the thiamine content of the various organs of the 15-day embryo when compared with those of the young chick. The present study was undertaken to determine the effect of incubation on the thiamine content of the egg. In order to do this, the entire content of the egg at various stages of development was analyzed.

While this study was in progress, the work of Westenbrink and Van Leer ('41) became available in abstract form. They approached the problem by studying separately the drop in the total thiamine in the yolk and the increase in thiamine content of the developing embryo. They concluded that a considerable part of the thiamine originally in the egg fails to appear in the embryo and is, therefore, apparently lost during development.

In our assays of the total thiamine content of the egg we have found no significant change in either fertilized or sterile eggs during the 3 weeks at 37-38°C. required for normal development.

<sup>1</sup> Aided by a Research Fellowship from Swift and Company, Chicago, Illinois.

## EXPERIMENTAL

The eggs from White Leghorn hens were supplied by Roger's Farms, a commercial hatchery. The eggs were weighed and placed in a Waring Blendor. The weights of the empty shells with their shell membranes were also recorded. After blending, 40 gm. were suspended in 250 ml. to give a convenient dilution for our assay procedure. At first solutions were made acid to litmus and autoclaved for 15 minutes at 15 pounds (Schultz, Atkin and Frey, '37). This resulted in some destruction of thiamine in the samples. Eventually it was found best to adjust the sample to pH 5.00 and autoclave for 10 minutes at 10 pounds. Samples thus prepared could be stored at 4°C. for several weeks without loss of thiamine activity.

All assays were carried out by the modification of the macro fermentation method of Schultz et al. ('42), as described by Scrimshaw and Stewart ('44). All values represent the average of at least two assay runs. When the agreement between these two runs was not satisfactory or when the values seemed to represent significant variations from the average, additional assays were carried out.

Four series of incubated eggs were studied utilizing eggs laid in May, 1943, January, February and April, 1944. Both sterile and fertile eggs were assayed in the first three series. Samples which were mixtures of four eggs were used throughout except for the study of the variation in individual unincubated eggs in series I. In series I and III controls were also kept at room temperature. Series III also had controls at 5°C. Series IV was planned primarily to check the previous findings on the thiamine content of fertile eggs during the last 5 days of incubation.

The variation in thiamine content of fresh hen eggs is shown in table 1. In series I the thiamine content of the whole egg varied from 62 to 93  $\mu\text{g.}/100\text{ gm.}$  When this was calculated on the basis of the total thiamine per egg, the variation was still from 35 to 56  $\mu\text{g. per egg.}$  Presumably the thiamine in the eggs varied more in the 1944 assays because the four egg samples of series II and III show about the same variation as the one egg samples of the previous spring. The average thiamine content was observed to vary from one series to the next. The May, 1943, (series I) eggs were distinctly lower in the thiamine content than those of the April, 1944 (series IV), being 79  $\mu\text{g.}/100\text{ gm.}$  as compared with 110 (table 2).

The results of assays on developing eggs are recorded in table 2. Although considerable variation was found within a series, no significant decrease in the total thiamine content of the egg was apparent. In

series I, II, and III the 20- and 21-day values are lower than the 15- and 18-day values. However, they are still within the range of variation seen in the unincubated samples. In series IV the 16- and 20-day values were checked by repeated assays on duplicate samples. The average values of 118 and 106  $\mu\text{g.}/100\text{ gm.}$  of whole egg fail to indicate a significant decrease in the total thiamine content.

TABLE 1

*Variation in thiamine content of fresh hen eggs.*

SAMPLE	WT. EGG MINUS SHELL	SERIES I SINGLE EGG SAMPLES		SERIES II 4 EGG SAMPLES	SERIES III 4 EGG SAMPLES
		Thiamine/100 gm. egg	Thiamine per egg	Thiamine/100 gm. egg	Thiamine/100 gm. egg
	<i>gm.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
1	52.7	93	56.0	94	81
2	50.0	62	35.2	75	84
3	55.0	70	44.1	95	70
4	45.3	86	45.2	100	88
5	51.2	77	45.4	76	103
6	47.5	89	48.1	88	74
7	49.3	85	48.5	..	85
8	52.2	73	43.8	..	86
9	49.0	80	45.4	..	85
10	54.8	77	47.9	..	..
Average	50.7	79	45.9	88	84

TABLE 2

*Thiamine content of fertile incubated eggs.*

DAYS IN- CUBATED	THIAMINE CONTENT PER 100 GM. OF EGG (SAMPLES OF 4 EGGS)			
	Series I	Series II	Series III	Series IV
	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>	<i>mg.</i>
fresh eggs	79	88	84	110
1	78	..	..	..
3	71	..	..	..
4	..	85	..	..
5	..	..	80	..
6	94	90	..	..
9	85	63	..	..
10	..	..	90	100
12	83	108	..	..
15	69	95	104	..
16	..	..	..	118
18	..	80	..	..
20	68	..	76	106
21	..	67	..	..

Similarly, no significant change could be detected in sterile eggs which were kept in the incubator with the developing ones (table 3). Control eggs kept at room temperature and at 5°C. likewise showed no decrease (table 3). The apparent increase in the thiamine content of the 20-day eggs in these cases is not significant.

TABLE 3  
*Thiamine content of non-developing eggs.*

DAYS STORED	THIAMINE CONTENT PER 100 GM. OF EGG (SAMPLES OF 4 EGGS)			
	Sterile incubated		Room temperature	Cold room 5°C.
	Series I	Series III	Series III	Series III
	$\mu g.$	$\mu g.$	$\mu g.$	$\mu g.$
0	79	84	84	84
3	82	..	..	..
9	72	..	..	..
12	70	..	..	..
15	78	92	85	78
20	80	100	112	100

#### SUMMARY AND CONCLUSIONS

The total amount of thiamine in hen eggs assayed by the macro fermentation method does not change during incubation. This differs somewhat from the conclusion of Westenbrink and Van Leer ('41) that thiamine disappears during incubation. The discrepancy probably lies in the fact that these workers assayed only the yolk and the embryo. Perhaps the thiamine content of the extra embryonic structures and fluids is sufficient to make up the difference reported by these workers.

It was also found that the thiamine content does not change in sterile eggs kept at incubation temperatures for 3 weeks or in eggs kept for this period at room temperatures and at 5°C.

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# THE EFFECT OF THIAMINE DEPLETION AND RESTORATION OF MUSCULAR EFFICIENCY AND ENDURANCE<sup>1</sup>

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## TWO FIGURES

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Since the early months of 1941 this laboratory has been carrying on experiments upon human subjects designed to bring out dietary factors which can influence to a measurable degree muscular efficiency or endurance or both. The Krogh bicycle ergometer and the Benedict Universal respiration apparatus provided the means of measuring the work done and the energy metabolism. One misses from most of the recent publications on this subject precise data on either of these items.

It was believed that the most favorable conditions for demonstrating alterations in efficiency and endurance would include the following: (1) The load and rate of work should be kept constant from day to day. (2) Likewise no dietary factor except the one under investigation should be allowed to vary significantly. (3) The subject should perform the identical work test time after time through, (a) a period of depletion of the vitamin by stages to very low levels until deterioration of performance became clearly evident. With intelligent and conscientious subjects one should not disregard subjective evidence of this state of deficiency and once it was established by unmistakable objective (and subjective) signs it should not be prolonged but should merge into (b) a period of gradual restoration until no further improvement could be discerned.

Any ideal series of experiments could readily be marred by the selection of uncooperative subjects or by an "edgy" relationship between operator and subject. The few men who figure most prominently in this particular study were selected from ten or twelve young men who were tried. Being of the more intelligent and more coopera-

<sup>1</sup>This study was supported by a grant from the Continental Baking Company of New York City and is taken from a thesis presented by J. W. A. in partial fulfillment of the requirements for the Doctorate of Philosophy.

tive type of students they were genuinely interested to learn their own capacities and to score a "perfect run" every day. They were also on the best of terms with the operator (J. W. A.) who encouraged them with praise when it was deserved and made them feel they were partners in the investigation.

The early papers on the role of thiamine in muscular work in man (McCormick, '40; Gounelle, '40; Morell, '40; Kraut and Droese, '41; Droese, '42 and others) apparently were optimistic to say the least. When a factor proves to be indispensable, a natural but uncritical attitude often is that above the level of mere sufficiency one should find more and more superior performance. Recent results have dispelled this concept concerning thiamine in relation to muscular work.

That thiamine is important in relation to exercise is seen clearly in the following investigations. Restriction of thiamine to 0.15 gm. daily for female patients in a state hospital, as reported by Williams, Mason, Wilder and Smith ('40, '42), produced after several months both physical and mental deterioration, the rate of appearance and severity of which definitely were augmented by physical exertion. Standardized tests on a chest exerciser were not borne so well as on a normal diet.

Further evidence of such a relationship is found in the work of Egaña et al. ('42) who investigated the effects on seven healthy physicians of a diet deficient in B-complex vitamins but adequate in calories and proteins for periods up to 4 weeks. They report moderate deterioration of physical fitness in exhaustive exercise and particularly poor recuperation between repeated periods of such work. The duration of an up-hill run which the subjects could tolerate was abnormally short in two of the three fittest subjects. At times the oxygen absorption was found to increase slightly as deficiency progressed and the authors inferred from this that the mechanical efficiency was impaired, but the authors did not center their attention on this phase of the work and apparently did not feel that impaired efficiency in the technical sense was demonstrated. Johnson et al. ('42) studied the effects of the same order of B-complex deficiency on ten men doing heavy labor. Marked deterioration in physical fitness made its appearance in 6 days. Brewer's yeast<sup>2</sup> in 18-gm. doses daily completely relieved all symptoms of deficiency and the physical deterioration within a week. The authors believe they definitely showed that thiamine alone of the B-complex factors will not maintain full physical efficiency.

<sup>2</sup> Fleischmann's no. 2019.

Of a quite different tenor are the experiments of Keys and Henschel ('41, '42) who have made extended observations on soldiers in which the amount of work seems to have been adequately measured but there is some doubt from their brief reports as published of the adequacy of measurement of respiratory metabolism. Their conclusion is that neither in brief, extreme exercise nor in prolonged, severe exercise and semi-starvation was there an indication of any effects, favorable or otherwise, of large daily supplements of thiamine chloride, riboflavin, nicotinic acid, pyridoxine, pantothenic acid, and ascorbic acid on muscular ability, endurance, resistance to fatigue, or recovery from exertion. In another communication Keys et al. ('43) concluded that no benefit of any kind was observed to be produced by intakes of more than 0.23 mg. of thiamine per 1000 cal. of the diet (0.7 mg. for the average person daily). Even at the lowest level, muscular, neuro-muscular, cardio-vascular, psycho-motor and metabolic functions were in no way limited.

A third group of workers, Foltz, Ivy and Barborka ('42) have made observations both on the effects of administration of extra B-complex vitamins and on depleted states (Barborka et al. '43). The first study demonstrated that intravenous injection of B-complex vitamins did not influence recovery from fatigue or increase muscular efficiency in persons on adequate diets. The second report showed that diets deficient in B-complex vitamins decrease work output in trained subjects; that early mild states of malnutrition with no objective evidence of deficiency disease do prevent maximum work efficiency; and that subjective symptoms of fatigue, irritability, lack of pep, anorexia and leg pains all disappeared and work output returned to normal or better within a few days following supplementation of the diet with B-complex vitamins (yeast concentrate).

#### EXPERIMENTAL

Experiments on the ergometer and respiration apparatus were conducted both in the post-absorptive and the post-cibal states. Briefly, each day's experiment consisted: (1) in determining the resting energy metabolism of the subject for a 10-minute period during which he sat without pedalling on the ergometer; and (2) in determining the metabolism for a 15-minute period of work and recovery, the subject producing work at the rate of 455-kg. meters per minute for 6 minutes of this period. This apportionment of time between active work and recovery from the oxygen debt was established after considerable study of the recovery of alveolar  $\text{CO}_2$  as well as pulse and respiration rates. Respiratory quotients were obtained and the ratio of work (in calories)

to energy expenditure due to work was calculated as net efficiency. If an endurance experiment was to be done on the same day it usually followed immediately after the muscular efficiency test. For the endurance test the subjects worked at rates varying between 1180- and 1475-kg. meters per minute, until they became exhausted, but did not breathe into the respiration apparatus. That the subject really was exhausted was readily established once the subject's characteristic performance was known.

Repeated checks were made of the Benedict Universal apparatus by combustion of alcohol in the system. Twenty such experiments gave an average R. Q. for ethyl alcohol of  $0.666 \pm .04$ .

The low vitamin diet used was selected especially for its low content of thiamine but, in all probability it was low in some of the other B-complex vitamins as well. A sample day's diet is given in table 1. The total number of calories was about 3000, and the percentages of protein, fat, and carbohydrate were 12, 41, and 47, respectively. Approximately 75 mg. of ascorbic acid were added to the diet daily. The estimated quantity of thiamine was 0.27 mg.<sup>3</sup> daily. A still lower thiamine diet (no. 3) was obtained later when the unenriched white bread was replaced with soda crackers. Analysis placed the level at 3 to 7  $\mu$ g. per 100 gm. of the food, which meant that the total day's supply of thiamine in the modified diet was less than 0.15 mg. Also no butter fat was included in the modified diet and this resulted in an increased carbohydrate percentage.

One hundred and eighty gm. of a peeled-wheat bread,<sup>4</sup> from flour produced by the flotation process of Earle (Murlin, Marshall and Kochakian, '41), were substituted first for the unenriched white bread in diet no. 2 when it was desired to add B-complex vitamins to the diet in a natural product. This bread contains a greater quantity of the vitamins than the usual "whole wheat" bread made from flour containing less of the whole grain (Sealock and Livermore, '43). Subsequently the peeled-wheat bread was doubled in quantity, and, during this period, the carbohydrate calories from other foods in the diet were decreased to compensate for the carbohydrate calories added by the extra 180 gm. of the peeled-wheat bread. The quantity of thiamine added by the substitution of the 180 gm. of the peeled-wheat bread was about 0.55 mg. daily, or 1.0 mg. from 360 gm. of the bread.

<sup>3</sup> All determinations of thiamine were made by Dr. N. S. Scrimshaw using the fermentation method of Schultz, Atkin and Frey ('42) as modified by Scrimshaw and Stewart ('44).

<sup>4</sup> This bread, known to the trade as "Staff," was supplied by the Continental Baking Company of Rochester, N. Y.

TABLE 1  
*Low thiamine diet no. 2.*

FOOD	Weight	Protein	CALORIES FROM Fat	Carbo- hydrate	Thiamine <sup>a</sup>
	(gm)				(μg)
Breakfast					
V.C.B. preparation <sup>1</sup>	10	..	..	25.0	..
White bread	60	22.3	3.8	102.7	27
Butter fat	25	..	198.0	..	..
Honey	33	0.4	..	55.0	6
Cream (40%)	100	9.8	165.6	11.5	..
Sugar	20	..	..	80.0	..
Egg white	90	49.2	..	..	..
Peaches	100	2.9	..	45.1	60
Coffee (1 cup)	..	..	..	..	..
Total		84.6	367.4	319.3	93
Lunch					
White bread	60	22.3	3.8	102.7	27
Butter fat	25	..	198.0	..	..
Salmon	100	38.6	108.0	..	..
Polished rice	55.6	18.2	1.4	180.4	18
Beets	100	7.0	..	41.0	18
Olives	40	16.4	81.0	15.6	..
Honey	33	0.4	..	55.0	6
Pears	100	3.6	8.0	217.2	38
Total		156.5	400.2	611.9	127
Dinner					
V.C.B. preparation	10	..	..	25.0	..
White bread	60	22.3	3.8	102.7	27
Butter fat	25	..	198.0	..	..
Sugar	30	..	..	120.0	..
Cream cheese	46	48.4	146.0	4.4	19
Cranberry jelly	50	..	..	143.5	..
Honey	33	0.4	..	55.0	6
Olives	40	16.4	81.0	15.6	..
Gelatin	5	20.5	..	..	..
Total		108.0	428.8	466.2	52
Grand total		349.1	1196.4	1397.4	272

<sup>1</sup> The V.C.B. preparation is a product manufactured by the Hülker and Bletsch Co., Chicago. It is stated as containing cane sugar, dextrose, concentrated fruit juices, essential oils, citric acid, artificial food color, and ascorbic acid, the latter in the amount of 3000 mg. for every 28 ounces, or about 3.8 mg. for every gram of the preparation.

TABLE 2

*Work metabolism data of L. H. during thiamine and B complex study.<sup>1</sup>*

DATE 1942	RESPIRATORY QUOTIENTS			Cc. CO <sub>2</sub> /cc. O <sub>2</sub> /MIN.		NET EFFICI- ENCY	ENDURANCE	
	Rest	Work and recovery	Excess	Rest	Work		%	kg. m.
Normal diet								
4/10	.81	.86	.89	206/254	1235/1415	18.39	4001	3' 23"
4/13	.87	.88	.89	198/229	1227/1387	18.01	4268	3' 36"
Average	.84	.87	.89	202/242	1231/1401	18.20		
Low thiamine diet								
4/14	.87	.95	1.00	216/249	1278/1310	19.74	4052	3' 26"
4/15	.96	.88	.83	231/242	1153/1347	19.57	4248	3' 36"
4/16	.79	.94	1.02	220/278	1197/1207	22.08	4858	4' 7"
4/17	.76	.91	1.01	211/277	1176/1233	21.62	4721	4' 0"
4/18	.92	.94	.95	218/238	1214/1283	20.11	5074	4' 18"
4/19	.83	.98	1.10	233/280	1246/1203	22.00	4937	4' 11"
4/20	.84	.96	1.03	222/263	1106/1121	23.90	4799	4' 4"
4/21	.73	.88	.97	189/259	1191/1290	20.10	4838	4' 6"
4/22	.81	.90	.96	206/254	1181/1271	20.64	4799	4' 4"
4/23	.80	.88	.94	197/247	1171/1287	20.32	4759	4' 2"
4/24	.83	.90	.95	206/248	1154/1250	21.02	5625	4' 46"
4/25	.88	.94	.98	199/226	1191/1242	20.58	5506	4' 40"
4/26	.83	.91	.95	215/258	1150/1238	21.38	5290	4' 29"
4/27	.88	.94	.97	212/241	1203/1265	20.51	5330	4' 31"
4/28	.85	.91	.95	223/260	1149/1232	21.57	5564	4' 43"
4/29	.82	.96	1.06	210/255	1177/1230	22.05	5605	4' 45"
4/30	.73	.91	1.03	190/262	1191/1238	20.83	5762	4' 53"
5/1	.69	.89	1.01	181/261	1211/1277	20.20		
5/2	.77	.84	.90	210/274	1078/1244	21.65	5099	5' 5"
5/3	.78	.88	.94	207/265	1147/1261	20.38	5447	4' 37"
5/4	.75	.89	.98	186/246	1199/1281	20.45	5564	4' 43"
5/5	.87	.90	.92	197/225	1195/1312	19.09	5447	4' 37"
5/6	.83	.92	.98	192/231	1190/1254	20.30	5389	4' 34"
5/7	.72	.90	1.01	174/242	1202/1258	20.73	5153	4' 22"
5/8	.85	.91	.94	213/249	1161/1260	20.84	5132	4' 21"
5/9	.87	.92	.92	214/245	1133/1211	21.46	4739	4' 1"
5/10	.82	.95	1.03	209/254	1267/1286	19.99	4642	3' 56"
5/11	.68	.92	1.04	155/228	1296/1323	19.49	4190	3' 33"
5/13	.73	.94	1.09	200/273	1265/1254	20.73	4246	3' 36"
5/15	.87	.95	1.02	215/247	1347/1289	21.28	3942	3' 32"
Average	.81	.92	.98	205/253	1194/1259	20.82		
Replacement of unenriched white bread with peeled wheat bread (180 gm.).								
5/16	.87	.87	.88	205/235	1141/1304	20.29	4097	3' 26"
5/17	.80	.82	.83	199/248	1063/1289	20.73	4284	3' 37"
5/19	.84	.93	.99	213/255	1150/1218	21.50	4223	3' 32"
5/20	.81	.90	.95	201/249	1164/1269	20.44	4187	3' 32"
5/21	.78	.95	1.06	199/256	1228/1227	21.01	4781	4' 4"
5/22	.82	.93	1.00	201/244	1233/1277	20.32	5346	4' 31"
5/23	.79	.98	1.11	193/246	1281/1230	20.66	5281	4' 25"
5/24	.72	.89	1.03	207/289	1168/1223	22.05	4804	4' 4"
Average	.80	.91	.98	202/253	1179/1255	20.88		
Peeled wheat bread doubled (360 gm.)								
5/25	.83	.93	1.00	214/259	1218/1261	20.81	4907	4' 8"
5/26	.84	.92	.96	218/258	1153/1218	21.50	5540	4' 43"
5/27	.88	.94	.97	208/236	1146/1201	21.42	6246	5' 13"
5/28	.94	.95	.95	217/230	1224/1289	20.16	6521	5' 36"
5/31	.73	.87	.95	177/243	1175/1292	19.73	7088	5' 55"
6/1	1.03	.95	.91	237/230	1132/1218	20.90	8566	7' 9"
Average	.88	.93	.96	212/243	1175/1247	20.75		

<sup>1</sup> All runs were made by this subject in the post-absorptive state.

Experiments were conducted with subject L. H., while on the low-thiamine diet no. 2, for a period of 28 consecutive days and 2 days at 1-day intervals (table 2 and fig. 1). At the end of this period the 180 gm. of peeled-wheat bread were substituted for the unenriched white bread in the diet and experiments were continued for 9 days. When the quantity of peeled-wheat bread was doubled, the experiments were continued for 8 days more. Corresponding periods for subject C. B.

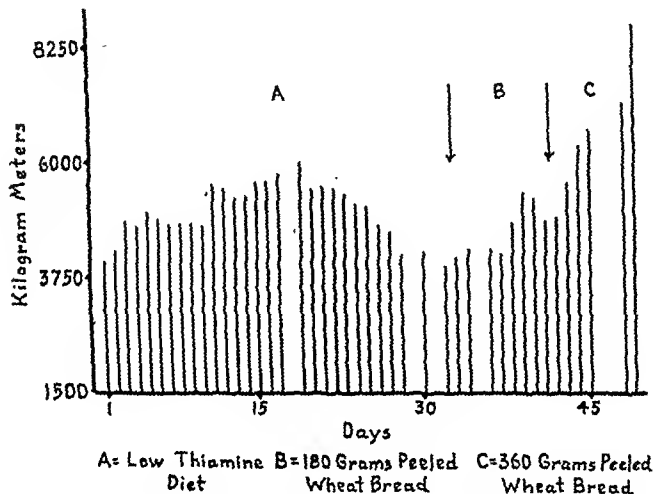


Figure 1

lasted 26, 8, and 7 days, respectively. Subsequent modifications in the study with C. B. consisted in the addition to the diet of thiamine hydrochloride, pyridoxine hydrochloride, and riboflavin for short periods (fig. 2). During the periods when the synthetic vitamins were added there were already 360 gm. of peeled-wheat bread daily in the diet. Experiments with L. H. were conducted in the post-absorptive state only, while those with C. B. were conducted in both the post-absorptive and the post-cibal states.

Subsequent studies were made when, for a period of 12 days, L. H. and C. B. went on the low thiamine diet no. 3 modified by the replacement of the unenriched white bread with soda crackers. Peeled-wheat



bread in the amount of 360 gm. was substituted for the soda crackers, and this resulted in a further increased carbohydrate content of the diet, as well as the addition of the B-complex vitamins. L. H. remained on this regime for 10 days and C. B. for 6 days. Later modifications in the diet of L. H. consisted in the addition of thiamine hydrochloride and a high vitamin yeast concentrate<sup>5</sup> for short periods. Finally 360 gm. of a high-vitamin yeast<sup>6</sup> bread, made from 20% soybean flour were substituted for the soda crackers for a short period. Efficiency measurements were conducted with both subjects in the post-cibal state. A third subject, J. S., was placed for 8 days on the low thiamine diet no. 3 used in these later experiments. Subsequently thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride separately were added to his diet for short periods, and measurements, including endurance experiments, were made.

### RESULTS

The muscular efficiency and endurance data, obtained in the first study with L. H., are given in full in table 2. It is evident from the table that efficiency and endurance increased—the former rather steeply but not smoothly for 7 days and the latter steadily during a period of 19 days (training effect) in spite of the deficiency; but from this point to the end of the low thiamine diet period, there was an equally steady decrease in the latter criterion of performance with no change in the former. The average net efficiency was 20.82%. The average net efficiency for the period when the 180 gm. of the unenriched white bread were replaced by the 180 gm. of the peeled-wheat bread was 20.88%, while endurance improved more than 25% in work and 1 minute in time. The average efficiency for the period when the quantity of peeled-wheat bread was doubled was 20.75%, but the endurance more than doubled over the deficiency period both in work and time. Thus the net efficiency, as measured during these post-absorptive experiments, remained practically the same, except for the first few days, during the entire 49 days, while endurance greatly improved after restoration of some factor or factors supplied by the peeled-wheat bread.

The same results were obtained in the post-absorptive experiments with C. B. as in those with L. H.: there was no increase in efficiency as a result of adding the B-complex vitamins in the form of the peeled-wheat bread. The average net efficiency for the low thiamine diet

<sup>5</sup> Fleischmann's.

<sup>6</sup> See Murlin, Marshall and Kochakian, '41, p. 579, for a description of this yeast.

was 19.79%, the average for the period with 180 gm. of the peeled-wheat bread substituted for the unenriched white bread was 19.12%, and the average with 360 gm. peeled-wheat was 19.20%. The inclusion in the diet of thiamine hydrochloride, pyridoxine hydrochloride, or riboflavin did not increase the efficiency in the post-absorptive state. Endurance, however, improved for the first 10 days (training effect) then declined, to a point much below the starting level, to the end of deficiency (fig. 2). Peeled-wheat bread raised it only for the first 3 days when fed at the lower level, and not so much as with L. H. at the higher

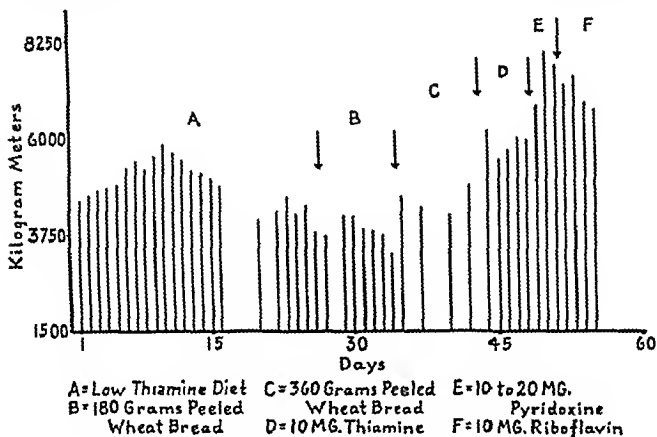


Figure 2

level. Addition of 10 mg. thiamine, however, raised endurance to a point equal relatively to that reached by L. H. on 360 gm. of the bread—a result indicating clearly that C. B. did not obtain the full benefit of the bread, or that his requirement for thiamine was much higher than that of L. H.

The average net efficiency of C. B. for the post-cibal, low thiamine experiments was 20.10% which was only a slight increase over that of the post-absorptive experiments. On 180 gm. of the peeled-wheat bread it was 21.27%, a relative increase of 5.5%; and when the peeled-wheat bread was doubled it was 20.95%, an increase of 4.2%. The addition of the synthetic vitamins resulted in no significant increases over the period when 180 gm. of the bread were ingested.

The post-cibal net efficiencies of C. B. are arranged in table 3 as "low" thiamine" values and "peeled-wheat-bread" values. The latter include all those post-cibal experiments in which either 180 or 360 gm. of the peeled-wheat bread were incorporated in the diet. The difference between the averages is statistically significant, and it appears that whole wheat bread contributes some factor affecting efficiency. That the increase was not due to the post-cibal state per se is indicated by the fact that the average net efficiency of the post-cibal experiments on the low thiamine diet was only slightly increased above the average efficiency of the post-absorptive experiments on the same diet. That the increase in efficiency was not due to the extra carbohydrate may be concluded, because carbohydrate foods were reduced in the diet to compensate for the extra starch contributed by the 360 gm. of peeled-wheat bread.

As the increase in efficiency with C. B. was of small magnitude, additional confirmatory experiments were conducted 6 months later when C. B. and L. H. were placed on the diet lowest in thiamine containing not more than 0.15 mg. daily. Experiments were conducted in the post-cibal (p. c.) as well as the post-absorptive state. Nearly all of the former were begun 1 hour after lunch. The average p. c. efficiency of L. H. on this diet was 21.05% (7 days). When 360 gm. of peeled-wheat bread (6 days), a yeast concentrate (1 day), and a high-vitamin yeast bread made with 20% soybean flour (2 days) were added separately to the diet, the average p. c. efficiency was 21.44%. The difference is not statistically significant. The average percentage p. c. net efficiency during the period of thiamine administration was 20.92%, practically the same as after lowest thiamine. The average p. c. efficiency of C. B., when the peeled-wheat bread was added to the diet, was 21.30%, in contrast with 19.68% on the low thiamine diet. This difference has statistical significance ( $p$  value 0.01) as in the former experiments with the same subject (table 3), when the  $p$  value was 0.02.

The average respiratory quotients of L. H. and C. B. during the thiamine and B-complex study are given in table 4. That carbohydrate oxidation was not decreased during the 32 days of the deficiency is evident from the excess respiratory quotients. A marked difference was apparent in the character of the metabolism during muscular work of the two subjects. The excess quotients of L. H. indicated almost pure carbohydrate combustion, while those of C. B. (average = 0.89) indicated that fat or protein contributed to the energy of work.

Some of the subjective symptoms noted by C. B. and L. H., while on the low thiamine diet, were fatigue, anorexia, slight diarrhea, in-

ability to concentrate well, and abdominal "fullness." These symptoms were observed first in about 10 days to 2 weeks of the deficiency. They progressed in intensity and at the end of the deficiency period nausea occurred in both subjects during the endurance rides. When 180 gm. of the peeled-wheat bread were added to the diet L. H. felt better but

TABLE 3  
*Post-cibal percentage net efficiencies of C.B.*

"LOW THIAMINE"	"PEELED WHEAT BREAD"
19.40	22.19
21.87	21.11
19.81	20.61
22.28	21.16
20.36	20.04
19.91	21.85
20.13	20.15
19.75	22.46
19.76	20.13
20.56	20.99
19.54	20.04
18.67	21.41
19.29	
Average	20.10 $\pm$ 1.00
	21.01 $\pm$ 0.85

Note — Fisher's *p* value of difference = .02.

TABLE 4  
*Average respiratory quotients of L.H. and C.B.*

PERIOD	REST R. Q.		WORK AND RECOVERY R. Q.		EXCESS R. Q.	
	L. H.	C. B.	L. H.	C. B.	L. H.	C. B.
1. Low thiamine diet no. 2 (0.27 mg. thiamine daily)	.81	.82	.92	.88	.98	.89
2. Substitution of 180 gm. of peeled-wheat bread (0.55 mg. thiamine extra)	.80	.79	.91	.85	.98	.89
3. 360 gm. of peeled-wheat bread (1.10 mg. thiamine extra)	.88	.87	.93	.86	.96	.87

C. B. did not. When 360 gm. of the bread were added both men felt decidedly better, and their mental attitudes improved.

The third subject J. S., rather corpulent in build but very willing and cooperative in temperament, participated in this study but his efficiency and endurance proved to be so low that only a short series

of experiments were run with him. Neither 10 mg. thiamine chloride nor 10 mg. riboflavin nor 25 mg. pyridoxine added as pure substances to the low thiamine diet raised his performance in any respect, probably because the earlier depletion period was not long enough.

#### SUMMARY AND CONCLUSIONS

1. Muscular endurance was greatly decreased on a diet which was low in the B-complex vitamins (thiamine 0.27 mg. daily). The effect was observed first in about 10 days to 2 weeks of the deficiency. When B-complex vitamins were added to the diet in the form of a natural source, such as a 98% whole wheat bread in amounts containing 0.55 and 1.10 mg. thiamine, the muscular endurance was increased significantly.

2. The inclusion of thiamine hydrochloride in pure form in a low B-complex diet resulted in marked improvement in endurance ability. Pyridoxine (in one experiment) had a similar effect, but riboflavin had none.

3. The inclusion of B-complex vitamins in a diet already adequate in these vitamins did not result in increased muscular endurance.

4. The muscular efficiency in the post-absorptive state remained the same for moderate work, even though the body was low in the B-complex vitamins, particularly thiamine, for a period of at least a month. The addition of B-complex vitamins, including thiamine, in such amounts that the daily requirements were met, did not increase the muscular efficiency in the post-absorptive state.

5. A small significant increase in muscular efficiency for moderate work was obtained with one subject in two different studies, when the B-complex vitamins were supplied to the diet by the peeled-wheat bread, and the experiments were conducted in the post-cibal state. A small increase, which was not significant, was obtained with another subject when a shorter series of experiments were conducted in the post-cibal state. It is concluded that any increase in efficiency which occurs with administration of the B-complex vitamins, on low B-complex diets of at least a month's duration, is in the post-cibal and not the post-absorptive state. This suggests that the entrance into the circulation and access to the muscles simultaneously of fuel and vitamins which promote release of energy are important. The administration of thiamine alone did not result in any increase in efficiency in the post-cibal state.

6. Carbohydrate oxidation, as a source of energy for moderate muscular work, was not decreased during a period of low B-complex intake for a period at least as long as a month.

7. The subjective symptoms of individuals on the diet, selected especially for its low thiamine content, and their improvement when B-complex vitamins were added to the diet in the peeled-wheat bread, have been noted as similar to those described by Williams, Wilder and associates ('40, '42). We confirm Johnson and associates ('42) in finding that the B-complex vitamins, given in these experiments as peeled-wheat bread, may improve efficiency and endurance whereas thiamine improves endurance only.

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# THE EFFECT OF INSTITUTIONAL COOKING METHODS ON THE VITAMIN CONTENT OF FOODS

## I. THE THIAMINE CONTENT OF POTATOES<sup>1</sup>

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Since a significant number of persons are eating daily one or more meals which have been prepared in large quantity, it is desirable to know the extent to which the nutritive value of food may be affected by the processes involved in institutional cooking. Accordingly, a study was undertaken to determine the losses of certain nutrients in foods prepared in large quantity.

The foods were obtained at the College dining hall which, at the beginning of the study, served three meals a day to approximately 500 college students. Six months later the students were replaced by 750 Army Air Corps students, the food services being taken over by the Army. The change in management did not necessitate a change in either personnel, equipment, techniques of cookery, or types of food served; therefore it was possible for the study to continue without interruption.

Inasmuch as the white potato forms a substantial part of the diet of many persons in the United States, it was one of the first vegetables studied. This paper reports data obtained on the thiamine content of the potato at different steps in preparation for serving.

### EXPERIMENTAL

The potatoes studied were of the Green Mountain variety, grown in the Connecticut River Valley. The analyses were begun in October, 1942, and carried on throughout the winter months, ending in May, 1943.

*Preparation of the potatoes.* The potatoes for both the noon and evening meal were peeled between 9 and 10 o'clock in the forenoon, approximately 500 lbs. being prepared each day. An electric abrasive peeler was used. The potatoes which were served for the noon meal

<sup>1</sup> Contribution no. 515 of the Massachusetts Agricultural Experiment Station.



were washed and cooked immediately, whereas those used for the evening meal were placed in a vat and soaked for approximately 6 hours in tap water, which was slowly running into and out of the vat. Upon removal from the water the potatoes were placed in perforated metal trays in a steam-oven and subjected to steam at a temperature of approximately 225°F. for 1 hour. During this cooking process whatever steam was condensed was automatically removed from the bottom of the steam-cabinet and discarded.

If the potatoes were to be mashed they were mashed immediately by whipping in an electric mixer. Fat, salt, pepper, and milk in the ratio of 5 lbs. to 50 lbs. of potatoes were added during the whipping. The entire mashing process took about 5 minutes.

During the interval between the completion of the cooking and the serving, the potatoes, either whole or mashed, were placed in trays in a steam warming-oven at a temperature of 157°F. The time of holding ranged from 50 to 120 minutes.

*Sampling.* Samples of the potatoes for analysis were taken at the following steps in preparation: (1) pared, (2) soaked 6 hours in tap water, (3) steamed, (4) mashed, (5) steamed and held for 1½ hours at 157°F. and (6) mashed and held for 1½ hours at 157°F. For the analysis of the whole potato, either in the raw or steamed condition, wedge-shaped slices about ¼ inch thick at the periphery were cut from the bud-end to the stem-end of the potato in order to obtain a representative sample from each tuber. For each sample slices were taken from 20 to 25 potatoes. These slices were cut in small pieces and aliquots taken for analysis. The mashed potatoes were sampled immediately upon the cessation of movement of the beater, small samples being removed from various areas until 600 to 700 gm. had been obtained.

All the sampling was done at the mess hall and the samples brought immediately to the laboratory where the analyses were carried out, the distance between the two buildings being about 300 feet. A period of approximately 15 minutes elapsed before the samples for thiamine determinations were placed in the extracting fluid. The weighings for the moisture determinations were made at the same time.

*Thiamine determination.* In the determination for thiamine the Hennessy and Cerecedo ('39) method as modified by Moyer and Tressler ('42) was used. For each sample 45 gm. of potatoes were taken and finely divided in a Waring Blendor with approximately 100 ml. of 0.1N H<sub>2</sub>SO<sub>4</sub>. The mixture was transferred to a 500 ml. flask and diluted with enough 0.1N H<sub>2</sub>SO<sub>4</sub> to bring the volume to 350-400 ml.;

it was then heated on a steam bath for  $\frac{1}{2}$  hour and later cooled to below 50° C. Twenty-five millimeters of 2.5M sodium acetate, containing 0.6 gm. of clarase and 0.4 gm. of papain were then added, and the sample was incubated at 47°C. for 15 hours. After incubation, the samples were cooled, made to volume, and filtered. The base exchange and oxidation procedures were carried out in the usual manner.

*Moisture.* Moisture determinations were made on all samples in an electric oven set at 80°C. Computation of the losses in thiamine values were based on the dry weight of the potatoes.

## RESULTS

*Effect of soaking.* The effect of soaking potatoes in running, cold water was studied in 9 lots of potatoes (see table 1). For the raw pared potatoes the thiamine content averaged 84 µg. per 100 gm.; whereas, following the soaking period it averaged 79 µg., indicating an average loss of 5%. That this loss was not due to chance alone, is seen in the statistical evaluation of the data (Student, '25), odds of 137 to 1 being obtained.

TABLE 1

*Effect of prolonged immersion in water on thiamine content of potatoes.*

LOT NO.	RAW, PARED			RAW, PARED, SOAKED			CHANGE IN THIAMINE CONTENT
	Moisture	Thiamine content		Moisture	Thiamine content		
		As exam.	Dry wt.		As exam.	Dry wt.	
		%	µg / 100 gm		µg / 100 gm	%	
1	81.7	85	464	81.5	78	422	- 9.0
2	82.0	82	455	81.3	77	412	- 9.4
3	81.4	80	430	80.8	80	417	- 3.0
4	82.1	87	486	82.0	86	478	- 1.6
5	84.2	69	437	82.7	69	399	- 8.7
6	82.4	90	511	84.3	78	494	- 3.3
12	80.0	81	405	81.0	77	405	0.0
13	81.0	83	441	80.7	86	443	+ 0.4
14	83.5	96	583	84.0	83	518	- 11.1
Ave.	82.0	84	468	82.0	79	443	- 5.1

*Effect of steam cooking.* The effect of steam cooking was studied in 6 lots of potatoes (see table 2). The thiamine content averaged 99 µg. per 100 gm., this value being somewhat higher than the value of 84 µg. found for the 9 lots of potatoes on which the effect of soaking was studied. Following cooking, the thiamine content had decreased in

each of the six instances to a mean value of 89  $\mu$ g. per 100 gm., the losses averaging 14.1%.

*Effect of mashing.* The effect of subsequent mashing on the thiamine content of the steam-cooked potato was studied in only 3 lots of potatoes; i. e., lots 8, 9, and 10. However, 5 other lots of potatoes (nos. 1 through 7) were analyzed for thiamine in both the raw and the mashed condition. The differences between the values for the 8 lots of the raw and the mashed potatoes represented losses which averaged 16.7%. This loss is slightly greater than the loss due to steaming alone. However, practically all of this difference may be accounted for by the dilution in thiamine which resulted from the addition to the potatoes of milk of lower thiamine content (35  $\mu$ g. per 100 ml.)

*Effect of holding in a steam-oven.* The influence of holding the potatoes, either whole or mashed, at a temperature of 157°F. for 1½ hours is also shown in table 2. The losses in the thiamine content of the whole potato averaged 8.3% whereas those for the mashed potato averaged 6.4%.

#### DISCUSSION

The mean value for the losses in thiamine in whole potatoes cooked and served under institutional conditions exclusive of losses during soaking was 20.5%, whereas that for the mashed potatoes was 21.7%, a difference which is not significant. The major loss accompanied the cooking of the potato. Additional losses were incurred during the preliminary soaking of the potato and during holding of the potato in the steam-oven until serving time 1½ hours later, the latter loss being approximately the same whether the potatoes were whole or mashed. It is probable that the mashing process was without effect on the vitamin.

The literature is not replete with papers dealing with the destruction of thiamine in potatoes during cooking processes. Only two studies have been found dealing with foods prepared on an institutional basis. Nagel and Harris ('43) determined the losses of thiamine in foods cooked in quantities sufficient to serve 600 persons at each meal. They reported a 5% loss in the thiamine content of the potato during cooking alone and a 29% loss due to the entire cooking process and subsequent holding on a steam-table for approximately 3 hours. Heller et al. ('43) on analyzing the thiamine in potatoes served in one of the cafeterias at the Brooklyn Navy Yard found a loss of only 2 to 7%. In contrast to these studies are four studies on potatoes prepared for serving under laboratory or home conditions. Thus Wiegand ('38) found losses

TABLE 2  
The loss in the thiamine content of potatoes due to steam-cooking, mashing and holding in a steam-oven.

LOT NO.	MOISTURE %	THIAMINE CONTENT			MOISTURE %	THIAMINE CONTENT				MOISTURE %	THIAMINE CONTENT			TOTAL LOSS IN THIAMINE %				
		As examined	Dry weight	μg/100 gm.		As examined	Dry weight	Loss %	As examined		Dry weight	Loss in steam-oven %						
													Raw, pared		Steamed 1 hr. at 225°F.	μg/100 gm.	Held in steam-oven 13 hrs. at 157°F.	μg/100 gm.
2 <sup>1</sup>	81.3	77	412	81.5	74	400	2.9	79.5	80	330	2.5	5.3						
5 <sup>1</sup>	82.7	69	399	82.0	62	346	13.3	80.1	62	317	8.1	20.3						
8 <sup>2</sup>	79.9	115	573	77.2	98	431	24.8	75.5	92	380	12.0	33.6						
0 <sup>2</sup>	80.7	103	533	79.7	100	493	7.5	78.8	90	419	11.8	21.4						
10 <sup>2</sup>	79.0	114	568	78.9	107	500	10.9	78.6	108	501	0.4	11.2						
11	79.9	114	568	78.8	90	426	25.0	78.5	84	301	12.1	31.1						
Ave.	80.7	99	509	79.7	89	434	14.1	78.5	86	400	8.3	20.5						
		Raw, pared			Steamed 1 hr. at 225°F				Steam-oven 13 hrs at 157°F									
1	81.5	78	422	82.0	74	411	2.6	79.1	81	390	5.1	7.6						
3	80.8	80	417	80.0	71	366	12.2	80.0	66	347	4.6	16.8						
4	82.0	86	478	81.5	83	450	5.8	80.9	82	430	4.4	10.0						
6	81.3	78	494	82.2	65	364	26.3	81.7	67	362	0.6	26.7						
7	82.4	78	443	80.9	69	261	18.5	79.5	67	257	9.4	26.2						
8 <sup>2</sup>	79.0	115	573	78.8	100	470	18.0	76.9	92	100	15.5	30.2						
9 <sup>2</sup>	80.7	103	533	77.5	94	419	21.4	79.3	81	291	6.6	24.6						
10 <sup>2</sup>	79.9	114	568	78.2	88	405	28.7	78.2	84	385	4.9	32.0						
Ave.	81.4	91	491	80.2	81	406	16.7	79.6	78	379	6.4	21.7						

<sup>1</sup> These potatoes were soaked 6 hours before cooking.

<sup>2</sup> These 3 lots of mashed potatoes were analyzed for thiamine at the raw, steam-cooked, and the mashed stages.

of thiamine due to cooking ranging from 15 to 75% and averaging 34%. Aughey and Daniel ('40) reported a 16% loss in baked potatoes and a 20% loss in boiled potatoes from which the cooking water had not been discarded. In boiled potatoes from which the cooking water had been discarded, Lane et al. ('42) observed a loss in thiamine approximating 36%. In the tentative report of the Food and Nutrition Board ('43) the values for losses in thiamine are given as ranging from 10 to 36% in boiled potatoes, 16 to 35% in baked potatoes and 10 to 55% for all methods combined.

The raw potatoes used in this study contained thiamine within the range of values reported by other investigators. Thus, Lane et al. ('42) found 84  $\mu$ g. per 100 gm. whereas Heller et al. ('43) reported values for five samples ranging from 53 to 90  $\mu$ g. Wiegand's data ('38) for five varieties grown in Holland indicate a range of values from 48 to 110  $\mu$ g. per 100 gm. Aughey and Daniel ('40) report, for two different samples of the Irish Cobbler variety, values of 135 and 150  $\mu$ g., per 100 gm., respectively. Booher et al. ('39) found newly harvested potatoes of the latter variety to contain as much as 186  $\mu$ g. per 100 gm. whereas old potatoes contained 147. In Nagel and Harris' ('43) study a value of 188  $\mu$ g. per 100 gm. was reported. On the basis of data obtained by the Bureau of Home Economics, the Food and Nutrition Board of the National Research Council ('43) has assigned to the edible portion of the white potato an average thiamine content of 90  $\mu$ g. per 100 gm., a value almost identical with that observed in this study, i. e., 89  $\mu$ g. for the fourteen lots of potatoes.

Obviously the potato does not rank with pork products, glandular meats, whole cereal grains, or legumes as a source of thiamine. Because of its high calorie content it would be rated by the Council on Foods ('39) as only a fair source of the vitamin. This rating however scarcely gives adequate credit to a food which comprises such a large proportion of the daily food as does the potato. The quantities of this tuber suggested by the Bureau of Home Economics ('41) for a low-cost, a moderate cost, and a liberal, adequate diet for a very active man would, at the lowest average value for thiamine obtained in this study, i. e., 78  $\mu$ g. per 100 gm., provide daily 0.38, 0.30 and 0.30 mg. thiamine or 16, 13, and 13%, respectively of the daily allowance (National Research Council, '43). Moreover the quantities of potatoes prescribed by the army (Howe, '42) would contribute 0.23 mg. or 11% of the total thiamine content of the rations. Certainly a food which could supply as much as one-sixth or one-ninth of the daily allowance of a nutrient must be considered a fairly significant source of that nutrient.

## SUMMARY

The thiamine content of Green Mountain potatoes has been studied at various steps in the cooking and serving procedures. The percentage loss in thiamine incurred at each step has been computed on a dry-weight basis. Small losses resulted from soaking the potato prior to cooking and from holding the potato in a steam-oven for a period as long as 1½ hours. The greatest loss, 14.1% of the original value for thiamine, occurred during the steam-cooking process. The mashing of the potato did not appear to be destructive of the thiamine. The over-all loss in thiamine exclusive of that during soaking approximated 20%.

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# REQUIREMENT OF TRYPTOPHANE BY THE CHICK

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## ONE FIGURE

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Previous reports on the tryptophane requirement of the chick have been made (Klose, Stokstad and Almquist, '38; Almquist and Meechi, '41). In the latter studies, acid-hydrolyzed casein supplemented with gelatin was used as the sole source of amino acids. Chick growth obtained with diets to which various levels of tryptophane had been added was used as an index to the tryptophane requirement, which was found to be approximately 0.5% of the diet. That this figure is probably too high was first suspected when analysis of casein by the method of Eckert ('43) yielded 1.2% tryptophane,<sup>1</sup> a value which would require feeding casein at 42% of the diet to provide sufficient tryptophane. It has already been shown that casein at the 20% level furnishes enough tryptophane for normal rate of gain (Almquist and Meechi, '41). Accordingly, the tryptophane requirement was investigated further with diets not employing acid-hydrolyzed casein. The two types of diet used in the present study were (1) a mixture of 19 amino acids to which varying levels of natural and synthetic tryptophane were added, and (2) a zein-gelatin diet to which several supplementary amino acids were added.

## EXPERIMENTAL

White Leghorn chicks were maintained from hatching time until 1 week of age on a practical rearing diet, at which time they were banded and weighed. Three days later they were weighed again, and on the bases of total weight, weight gain, and vigor, were segregated into groups of 2 to 5 chicks and housed in electrically heated, wire-floored battery brooders.

The diets were fed ad libitum for 12 days. The basal ration for the amino acid mixture diets was as follows: cellulose<sup>2</sup> 5, calcium gluconate 8, mineral mixture 3.24, crude soybean oil 5, sardine oil (400D-3000A

<sup>1</sup> We are indebted to Dr. F. H. Kratzer for permission to cite this result from his analyses.

<sup>2</sup> Cellu Flour.



per gram) to which 1% of mixed tocopherols<sup>3</sup> had been added 0.25, choline chloride 0.2, inositol 0.1, cholic acid 0.1, 2-methyl-1, 4-naphtho-hydroquinone diacetate 0.001, thiamine 0.001, riboflavin 0.001, pyrid-oxine 0.001, nicotinic acid 0.003, calcium pantothenate (*dl*) 0.003, biotin (acid)<sup>4</sup> 0.00001 gm., with the amino acid mixture and glucose<sup>5</sup> to make 100 gm. Other members of the vitamin B-complex were provided by a concentrate prepared from solubilized liver which added only 4 mg. nitrogen to each 100 gm. diet. The mineral mixture furnished the following substances per 100 gm. of diet: tricalcium phosphate 2000, dipotassium phosphate 500, potassium chloride 300, and manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8, and cobalt 0.5 mg.

All of the amino acids in this diet were commercial products, and as far as possible, were from the same lot throughout the experiments. Data as to the levels used and the form in which the acids were added are summarized in table 1. The calculated amount of sodium bicarbon-

TABLE 1.  
*Mixture of amino acids used in the basal diet.*

AMINO ACID	FORM OF ACID	LEVEL AS FED	LEVEL OF NATURAL ISOMER
		%	%
Alanine	dl	1.0	0.5
Arginine	1 (+) HCl	1.4	1.2
Aspartic acid	1 (+)	2.0	2.0
Cystine	1 (-)	0.4	0.4
Glutamic acid	1 (+)	5.0	5.0
Glycine	—	1.3	1.8
Histidine	1 (+) HCl H <sub>2</sub> O	0.8	0.6
Hydroxyproline	1 (-)	0.2	0.2
Isoleucine	dl	2.0	1.0
Leucine	1 (-)	2.0	2.0
Lysine	1 (+) HCl	1.4	1.1
Methionine	dl	1.0	0.5
Norleucine	dl	0.2	0.1
Phenylalanine	dl	1.0	0.5
Proline	1 (-)	2.0	2.0
Serine	dl	0.4	0.2
Threonine	dl	3.0	1.5
Tryptophane	..	..	..
Tyrosine	1 (-)	2.0	2.0
Valine	dl	2.0	1.0
Total		29.6	23.6

<sup>3</sup> Natural mixed tocopherols, Type I (Distillation Products, Inc.).

<sup>4</sup> The biotin was kindly provided by Merek & Co., Inc., through the courtesy of Dr. J. C. Keresteszy.

<sup>5</sup> Cerelose.

ate was added to neutralize the hydrochlorides of the basic amino acids, and to provide sodium chloride, which was omitted from the mineral mixture generally used.

Except for the amino acid source, the zein basal diet was the same as the amino acid basal, with the nitrogen supplied by the following materials in %: zein 20, *l* (—)-cystine 0.3, *l* (+)-lysine 0.4, *dl*-methionine 0.2, *dl*-valine 1.0, *dl*-threonine 2.0 and gelatin (or fibrin) 10. Fibrin was used at a 10% level in one zein diet as a positive control for the completeness of the total protein source, as well as for the tryptophane, which it supplied at 0.32% of the diet.<sup>1</sup> When gelatin was used with zein, the diet contained less than 0.04% tryptophane. The supplements of natural and synthetic tryptophane<sup>6</sup> which were used were the same for all experiments. The specific rotation of the *l* (—) form was  $-31.2^\circ$  at  $20^\circ\text{C}$ .<sup>7</sup>

### RESULTS

The best rates of growth obtained in these studies were approximately 5% per day for a 10-day period, which is slightly less than that obtained with a methionine-supplemented soybean protein as the sole protein source for the chick (Grau and Almqvist, '43). With the amino acid mixtures, however, some chicks did not eat the diets readily for the first day or two. Because of the variability of the early growth period with its consequent effect on the rate of gain, the efficiency of gain (gm. gain/gm. feed consumed) has been used, instead, as an index of the tryptophane requirement. Better agreement was obtained between experiments and between the two types of diets when the results were evaluated in this way. In both types of diets the maximal efficiency of gain was reached when 0.2 to 0.3% *l* (—)-tryptophane was present. This is approximately the amount furnished by 20% of intact casein, which is known to be an adequate source. The results of the various experiments are presented in table 2. The efficiency of gain has been plotted against the addition of tryptophane in figure 1. Curve A is based on average values of gain/feed for all experiments plotted against *l* (—)-tryptophane added. Consideration of curve B shows that the requirement for tryptophane in the *dl* form is twice that for the natural form, and indicates that the chick does not make appreciable use of the *d* (+) form for growth. From curve A it is estimated that the *l* (—)-tryptophane requirement of the chick is approximately 0.25% of the diet for maximal efficiency of utilization of

<sup>6</sup> Merck & Co., Inc.

<sup>7</sup>  $[\alpha]_D^{20}$  for pure *l* (—)-tryptophane is given as  $-31.5^\circ$ . (Greenberg, '44).

TABLE 2

*Relation of added natural and synthetic tryptophane to efficiency of feed utilization.*

DIET NO.	NO. CHICKS PER GROUP	TYPE OF DIET	TRYPTOPHANE ADDED		GAIN PER GM. FEED CONSUMED
			l (—)	dl	
1	2	Amino acid mixture	%	%	gm.
			0	0	— 0.32
			0	0.1	— 0.07
			0.1	0	+ 0.08
			0	0.2	+ 0.08
			0.2	0	+ 0.33
2	4	Amino acid mixture	0.1	0	+ 0.17
			0	0.2	+ 0.06
			0.2	0	+ 0.29
			0	0.4	+ 0.27
			0.3	0	+ 0.30
			0	0.6	+ 0.31
			0.4	0	+ 0.31
			0	0.8	+ 0.30
3	5	Zein + gelatin	0	0	— 0.45
			0.1	0	+ 0.17
			0.2	0	+ 0.27
			0.3	0	+ 0.33
			0.6	0	+ 0.29
4	5	Zein + fibrin	0.32	0	+ 0.33

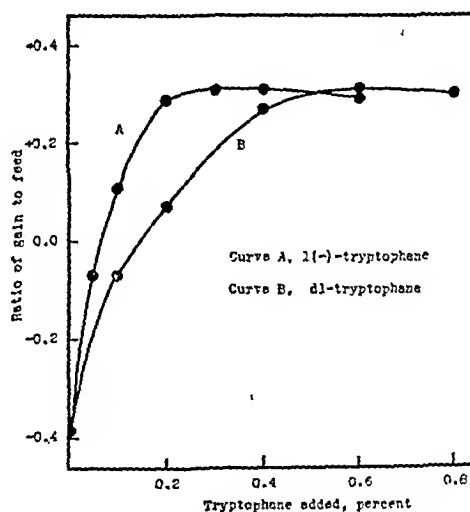


Fig. 1 The effect of the per cent of l (—)- and dl-tryptophane added to the diet on the ratio of chick weight gain to feed eaten.

the diet. Maximal rates of increase in body weight were also attained at approximately this level of *l* (—)-tryptophane.

Since the requirement previously suggested was approximately twice the value reported here, it seems possible that the isolated, natural tryptophane used in the former studies may have been largely racemized. The present results reveal a further difference between the amino acid requirements of the chick and of the rat, for the latter animal can apparently utilize both isomers of tryptophane for growth (Berg and Potgeister, '31-'32; du Vigneaud, Sealock, and Van Etten, '32; Berg, '36). In the mouse less rapid growth is obtained with the unnatural form than with the natural form (Totter and Berg, '39).

### SUMMARY

Use of a diet containing a mixture of 20 amino acids or one containing zein and gelatin supplemented with some pure amino acids as the only protein source has shown *dl*-tryptophane to be only half as effective as *l* (—)-tryptophane in promoting growth and efficient food utilization in the chick. From the results obtained with these diets, the *l* (—)-tryptophane requirement is shown to be approximately 0.25% of the diet.

### ACKNOWLEDGMENT

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# THE FRACTIONATION OF PHOSPHORUS COMPOUNDS IN CERTAIN VEGETABLES<sup>1</sup>.

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Phosphorus compounds in plant and animal tissues and fluids, so far as known, are formed from ortho-phosphoric acid. The capacity for the characteristic reactions is dependent upon the three hydroxyl groups. They not only react with other compounds but they may also react with other molecules of phosphoric acid giving rise to di- or tri-phosphoric acid compounds such as one finds in the nucleotide adenosine. Since phosphoric acid is capable of forming many more or less distinct groups of substances with many functions (James and Arney, '39; Peters and Van Slyke, '31) different concentrations of some fractions should be found in plants having different metabolic patterns. Such information may be of value in understanding further the role of the element once referred to as "phosphorus mirabilis" (Thorpe, '28). Furthermore, since vegetables may furnish an increased proportion of our phosphorus requirements it is desirable to determine whether or not the organic phosphorus exists in available forms in certain foods (Lowe and Steenbock, '36; Lowe, Steenbock and Krieger, '39; McCance and Widdowson, '35; Yang and Dju, '40).

This paper therefore presents a quantitative fractionation of the phosphorus compounds of eleven common vegetables into the following groups: inorganic, organic, resistant esters, phosphoprotein and phospholipid phosphorus, and phytin.

## EXPERIMENTAL

The values reported for each vegetable represent the average of duplicate or triplicate determinations on one sample which was prepared from several kilograms of representative fresh material.

The scheme of separation was similar to that used by Arney ('39). All manipulations were checked for the recovery of phosphorus by the use of solutions of known phosphorus content. All phosphorus determinations were made colorimetrically by the Truog and Meyer method ('29). Total phosphorus was brought into solution by wet ashing

<sup>1</sup> Contribution no. 529 of the Massachusetts Agricultural Experiment Station.

with sulphuric and nitric acids. Resistant esters and inorganic phosphorus were determined on clarified trichloroacetic acid extracts. Extractions with trichloroacetic acid made over periods of 30, 60 and 90 minutes indicated that an end point was reached in 30 minutes. The original solutions were too colored to use directly, hence all phosphorus compounds were precipitated by a procedure similar to that of Needham, Needham, Baldwin and Yudkin ('32). The precipitate was dissolved in an acid solution of pH 1. The inorganic fraction was determined directly on this solution, while a total phosphorus determination was also made by wet ashing the extract, in order to obtain the resistant esters by difference. Phospholipid and phosphoprotein phosphorus constituted the difference between the total phosphorus and the acid soluble phosphorus. Phytin phosphorus was determined by the McCance and Widdowson method ('35).

The results of the investigation are shown in table 1.

TABLE 1

*The percentage content of phosphorus on a dry matter basis in various forms in certain vegetables and their percentage distribution.*

VEGETABLE	TOTAL	INORGANIC	ORGANIC	RESISTANT ESTERS	PHOSPHO- LIPIDS AND PHOSPHO- PROTEINS
Asparagus, <i>Asparagus officinalis</i> L.	0.699	0.359 (51.4) <sup>1</sup>	0.340 (48.6)	0.184 (26.3)	0.156 (22.3)
Cabbage, <i>Brassica oleracea</i> L.	0.414	0.256 (61.8)	0.158 (38.2)	0.080 (19.3)	0.078 (18.9)
Carrot, <i>Daucus carota</i> L.	0.350	0.247 (70.6)	0.103 (29.4)	0.059 (16.9)	0.044 (12.5)
Dandelion, <i>Taraxacum officinale</i> W.	0.439	0.257 (58.5)	0.182 (41.5)	0.068 (15.5)	0.114 (26.0)
Eggplant, <i>Solanum melongena</i> L.	0.271	0.196 (72.3)	0.075 (27.7)	0.040 (14.8)	0.035 (12.9)
Kale, <i>Brassica oleracea</i> L.	0.437	0.256 (58.6)	0.181 (41.4)	0.062 (14.2)	0.119 (27.2)
Spinach, <i>Spinacia oleracea</i> L.	0.873	0.568 (65.1)	0.305 (34.9)	0.190 (21.8)	0.115 (13.1)
String beans, <i>Phaseolus vulgaris</i> L.	0.505	0.357 (70.7)	0.148 (29.3)	0.044 (8.7)	0.104 (20.6)
Squash, <i>Cucurbita maxima</i> D.	0.423	0.213 (50.4)	0.210 (49.6)	0.029 (6.9)	0.181 (42.7)
Turnip, <i>Brassica rapa</i> L.	0.543	0.335 (61.7)	0.208 (38.3)	0.120 (22.1)	0.088 (16.2)
Onions, <i>Allium cepa</i> L.	0.291	0.185 (63.6)	0.106 (36.4)	0.087 (29.9)	0.019 (6.5)

<sup>1</sup> The figures in parentheses indicate the percentage of the total phosphorus which each fraction represents.

Inorganic phosphorus ranged from about 50 to over 72% of the total. Resistant esters averaged slightly less than the phospholipid and phosphoprotein phosphorus; the former varied from about 7 to 30%, the latter from 6.5 to 42.7%. Phytin was absent in all cases.

### SUMMARY

From these data one may conclude that the bulk of the phosphorus is in the inorganic form; that the organic fraction may be composed of widely varying amounts of resistant esters, and the phosphoproteins and phospholipids; and that there are no unavailable forms of phosphorus present in these vegetables.

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# EFFECTS OF VARIATIONS IN DIETARY PROTEIN ON THE PHYSICAL WELL BEING OF MEN DOING MANUAL WORK<sup>1</sup>

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## INTRODUCTION

The question of the effects of low and high protein diets has become an immediately practical one, not only because of the relative scarcity of high protein foods but also because of the greater difficulty in shipping them long distances. Previous work has shown clearly what was the minimum protein intake necessary to maintain nitrogen equilibrium (Reviews — Lusk, '31 and Terroine, '36) and that low levels are compatible with health in sedentary men. Although frequent statements are to be found that higher intakes are necessary for hard work, almost none of these is supported by experimental proof. In fact the most extensive experimental work, that of Chittenden ('04), reported increased vigor for hard working men when the protein intake was lowered to 50-60 gm.

In order to make the results of this work useful for direct practical application, the aim was to utilize normal young men in a natural environment carrying on their usual regime of work. For the same reason the diets were planned to include only items to be found in normal diets, and thus to avoid synthetic or laboratory foods which would not be encountered in practical dietary experience. The plan in one group of subjects was to restrict protein foods as much as possible within the above limitations; in another group of subjects to give the greatest possible excess of high protein foods. In a third group the individuals were to continue on their usual diet.

The conclusions from this work must be limited to results appearing within the time limit of the tests, namely 8 weeks of the modified diet.

<sup>1</sup>The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

Therefore, the practical implications must likewise be limited to apply to emergency modifications of diet lasting up to 2 months.

#### METHODS

The subjects were twenty-four volunteers from the personnel of a Civilian Public Service Camp, who continued on the work regime of the camp and ate their meals at special tables in the regular mess hall. At the start each subject was given a thorough physical examination, clinical laboratory examination of blood and urine and blood chemical estimation of NPN, protein and A/G Ratio. NPN was estimated by the method of Daly ('33); serum protein and urinary nitrogen by the Micro Kjeldahl method of Ma and Zuazaga ('42); and serum protein fractionation by the method of Howe ('21). Throughout the study frequent check-up physical examinations were held and the estimations of blood NPN and protein were repeated weekly along with a measurement of the nitrogen in a 24-hour specimen of urine. Assessment of physical fitness each week was by means of the so-called "Pack Test." In this test the subject, wearing a rucksack weighted to approximately  $\frac{1}{2}$  his body weight stepped up onto a 16-inch platform and back down onto the floor once each 2 seconds (handrails at shoulder height were used as an aid). The stepping exercise was terminated at 5 minutes if the subject could carry on that long; after stopping, the pulse was counted for three periods: 1 to  $1\frac{1}{2}$  min., 2 to  $2\frac{1}{2}$  min. and 4 to  $4\frac{1}{2}$  min. of the recovery period. From these data a numerical score was calculated as:

$$\frac{\text{duration exercise in seconds}}{2 \times (\text{Sum of pulse beats counted in the three periods})}$$

This test had been found previously to correlate with physical ability in athletes and to parallel improvements with training and deterioration accompanying proven inadequate diets. In order to have an additional index of recuperative powers, the test was administered twice on each occasion with only 15 minutes rest between the two.

The dietary test program consisted of three periods — 2 weeks preliminary control, 8 weeks of modified diet and 2 weeks of final control. The twenty-four subjects were divided in three groups of eight, each group including a rough cross section of the whole in respect to occupation and physical fitness. All groups ate the regular camp diet for the first 2 weeks and one of the groups of eight continued thus throughout. A second group, called the restricted protein group, changed their diet at the end of the first control period to one which allowed no meat,

cheese, eggs, nuts, legumes and only up to  $\frac{1}{2}$  cup of milk daily. Bread was the only other food allowed the restricted protein group which contained animal protein (milk); this was limited in amount to this group so that not more than  $2\frac{1}{2}$  gm. of milk protein was furnished daily in this form and not more than 5 gm. of animal protein in the entire daily diet (the other  $2\frac{1}{2}$  gm. or less in top milk or cereal). Unlimited amounts of low protein foods were furnished this group and they, like the other groups, were urged to fully satisfy their appetites. During the final control period this group was given a high protein diet.

The third group, called the high protein group, was furnished with large amounts of high protein foods and allowed only restricted amounts of low protein foods.

All groups were given daily 5 gm. of yeast extract<sup>2</sup> fortified with additional riboflavin to forestall any possibility of vitamin B deficiency. As will be noted from the above description, the dictates of taste were followed in so far as possible in this type of experiment. For measurements of the diet each mess table was furnished with a diet scales and standard ladles. All articles of food were furnished in standard portions of known weight or else the subjects weighed the portion eaten. Each subject had a special record book with a page for each day in which he noted the amounts of each article of food eaten during the day on one side of the sheet and the day's activities hour by hour on the other side. These books were kept in a rack in the dining room. The proper analytic figures for protein, fat and calories were entered in these books periodically by the dietitian staff. All items in the diet known to contain significant amounts of protein were analyzed at least once for protein during the experiments. Those subject to variation (e.g., bread, cereals) in cooking were analyzed each time they were on the menu. Our own analytic figures were supplemented with those of Bowes and Church ('40) for fat and calories and for the traces of protein in low protein foods. Whenever possible the manufacturer or haker was consulted to determine ingredients.

The work of the subjects included office work, kitchen work, laundry work, farming and forestry work (including work on trails, clearing forest ground and repair of roads). The daily calorie expenditure, depending on the subject's job ranged from 2400 to 5000 cal. with an average of approximately 3300.

<sup>2</sup> Standard Brands Co., Type 3. This amount of fortified yeast contained approximately 5 mg. thiamine, 1.5 mg. riboflavin and 10 mg. niacin.

## RESULTS

*I. Restricted protein group*

The averages of daily protein intakes in this group of eight are shown in table 1 along with the caloric intakes, subjects' weights and their usual occupations. It will be seen that even with the severe restriction of high protein foods the daily protein intake was rarely below 50 gm. and averaged 53 gm. When the caloric intake was above 4000 cal. the protein was usually nearer 60 gm. Potato, bread and other cereal products were the chief sources of this protein; less than 5 gm. was of animal origin. Two subjects of this group terminated their experiment early, one because of an acute attack of recurrent appendicitis, the other because of transfer to another project.

The 24-hour urinary excretions of nitrogen were measured once a week and the results, expressed as grams of equivalent protein, are included in table 1. Since these figures are calculated from samples taken only once weekly whereas the figures for intake were obtained daily, exact agreement should not be expected. However, it can be seen that the urinary nitrogen figures furnish a rough check on the calculated intake, if the usual assumption of 1 gm. of stool nitrogen is made. If anything, the intake figures tend to be a little larger than the corrected urinary figure so it may be safely stated that the calculated mean daily intake figure of 53 gm. is perhaps too high.

In general all the subjects in this group believed they were getting enough food. It was their observation, however, especially those doing the heaviest work, that they became unusually hungry and felt a little weak late in the morning and late in the afternoon. There was an average weight loss during the 8-week period of 0.9 kg., a good part of which could be logically explained by the fact that several of the group started on a regime of much heavier outdoor work than was customary to them. A weight loss of similar magnitude was found in the group on the normal diet. One man who lost 2.8 kg. was obese and rather "flabby" at the start.

Aside from the experiences of late morning and afternoon hunger mentioned above, none of the subjects complained of unusual symptoms. They were able to carry on the work routine; no complaints by their work supervisors were ever made. The pack test data confirms the impression that they showed no deterioration. Table 2 summarizes these results on this group and those on the normal and high protein diets. In spite of some irregularities it will be seen that both groups improved slightly; a few individuals strikingly, undoubtedly

TABLE 1  
Summary of caloric intakes, protein intakes and urinary nitrogen excretions expressed as "equivalent protein".

EXPERIMENTAL SUBJECTS				PRELIMINARY CONTROL PERIOD (DAILY)			EXPERIMENTAL PERIOD						TOTAL CONTROL PERIOD (DAILY)		
Diet	Individual	Occupation	Body weight kg.	Caloric intake <sup>1</sup> gm.	Protein intake <sup>1</sup> gm.	Protein metabolism <sup>2</sup> gm.	1st four weeks (Daily)			2nd four weeks (Daily)			Caloric intake <sup>1</sup> gm.	Protein intake <sup>1</sup> gm.	Protein metabolism <sup>2</sup> gm.
							Caloric intake <sup>1</sup> gm.	Protein intake <sup>1</sup> gm.	Protein metabolism <sup>2</sup> gm.	Caloric intake <sup>1</sup> gm.	Protein intake <sup>1</sup> gm.	Protein metabolism <sup>2</sup> gm.			
Normal	Co	Forestry	71.1	2836	100	..	3870	125	102	..	..	..	3350	114	..
	Ik	Truckdriving	72.0	3140	110	108	3640	115	81	3340	108	91	2960	97	77
	K	Camp maintenance	75.0	2720	94	72	2830	101	80	2920	107	99	2790	97	79
	Lar	Kitchen	77.0	3080	123	..	2750	102	68	3460	131	71	2970	92	104
	M	Carpentry	72.3	3550	128	102	3460	107	92	3140	105	94	2970	92	104
	N	Carpentry	72.8	3460	123	102	2060	71	63	2280	76	61	..	..	..
	N	Kitchen	72.8	2460	81	85	3020	123	98	3130	108	80	3000	92	87
Restricted protein	Su	Office	68.3	3590	126	104	3030	123	98	3380	102	85	3190	95	64
	Tr	Carpentry	73.8	3040	101	79	3150	100	75	3380	102	85	3190	95	64
	Average		72.9	3050	108	92	3170	106	82	3080	105	83	3050	98	80
	B	Truckdriving	85.0	3090	110	88	3300	55	42	3230	57	41	4440	189	128
	F	Kitchen	79.7	3240	103	100	3750	57	45	3800	53	40	4110	185	141
	G	Road repair	59.2	2770	75	58	3020	52	41	3230	52	36	3810	135	114
	J	Office	87.7	2960	83	67	3140	56	54	..	..	..	..	..	..
High protein	R	Farming	69.7	3680	125	127	3750	57	66	4300	59	47	5600	274	207
	Su	Road construction	85.9	3490	117	108	3470	57	51	..	..	..	..	..	..
	Stc	Kitchen	74.9	2800	92	96	2960	39	43	2950	43	34	3710	184	73
	Stc	Forestry	63.3	3560	122	99	3170	48	44	3350	50	41	4160	198	131
	Average		76.9	3180	103	93	3230	53	48	3480	53	40	4310	194	132
	A	Office	60.0	2080	78	01	2330	96	83	2380	110	88	2340	89	69
	C	Farming	68.9	3660	142	120	3680	130	150	3980	189	179	3150	125	117
Average	D	Kitchen	107.0	2460	103	98	3290	139	128	3590	182	144	2750	100	123
	Li	Office	83.2	2980	97	75	3660	153	122	3770	159	115	3150	104	103
	Lo	Forestry	80.0	3810	126	112	4440	193	104	3810	172	163	3150	112	107
	Sn	Forestry	84.3	3410	114	..	3950	174	161	5010	254	153	3290	137	124
	Tu	Office	72.4	2980	108	104	3270	151	132	3820	170	135	2380	82	68
	W	Laundry	62.6	3240	114	98	4430	185	156	4090	175	159	3060	106	98
	Average		78.6	3080	110	95	3670	160	138	3810	177	142	2910	107	101

<sup>1</sup> Average values calculated from weights of food eaten and analytic figures (all days calculated).<sup>2</sup> Average values from analysis for nitrogen of 24-hour urine, collected only once weekly (gm. nitrogen x 6.25 to obtain figure in gm. protein for comparison with intake).

due chiefly to the physical training from their active work regime. The irregularities of scores are in some cases due to changes in motivation on different days; in other cases to local disabilities arising from minor injuries. The relative scores on the two consecutive tests on the same day did not yield any additional information and are not reported in detail. It was an unexplained fact that the relationship of the second to the first score was largely characteristic of the individual: many dropped off slightly every time on the second score, a few markedly, while a few actually improved in every instance.

TABLE 2

*Physical fitness as indicated by scores in "park test".*

DIET	SUBJECT	PRELIMINARY CONTROL PERIOD: WEEK		EXPERIMENTAL PERIOD: WEEK								FINAL CONTROL PERIOD: WEEK	
		1st	2nd	1st	2nd	3rd	4th	5th	6th	7th	8th	1st	2nd
Normal diet	Co	71	..	81	78	72	..	..	..	..	..	..	..
	H	105	..	92	99	110	110	102	103	113	102	108	106
	K	57	50	55	61	62	58	67	65	58	68	65	52
	Lar	31	..	52	40	37	81	79	88	86	68	89	69
	M	43	78	85	86	96	102	96	87	87	88	106	99
	N	70	58	53	47	39	31	48	53	52	61	..	..
	Su	46	82	77	81	77	73	83	85	86	73	86	91
	Tr	..	..	62	67	74	85	88	86	86	85	89	92
Average increase <sup>1</sup>				..	- 6	- 4	+ 1	+ 5	+ 6	+ 6	+ 3	+ 15	+ 9
Restricted protein	B	26	23	27	34	37	34	38	42	48	85	54	79
	F	68	77	84	80	77	82	95	91	95	84	89	87
	G	48	75	85	85	96	77	82	88	90	84	83	..
	J	30	45	53	48	30	44	..	..	..	..	..	..
	R	81	82	82	..	..	82	73	80	82	..	82	79
	Sm	56	58	79	..	87	85	90	..	..	..	..	..
	Ste	67	87	88	95	89	103	98	107	88	90	99	86
	Sto	92	93	111	102	97	93	96	101	111	101	106	98
Average increase <sup>1</sup>				..	- 1	- 2	- 1	+ 2	+ 6	+ 6	+ 10	+ 6	+ 7
High protein	A	71	69	73	68	77	70	84	83	79	84	83	80
	C	84	87	92	90	90	92	96	92	94	85	88	91
	D	32	37	52	35	48	47	54	62	55	38	50	53
	Li	69	..	83	94	87	80	84	78	86	79	93	87
	Lo	97	87	94	89	93	98	85	82	91	87	89	80
	Sa	109	88	..	100	..	107	104	108	111	108	105	134
	Tu	77	81	83	80	79	83	83	83	87	87	79	83
	W	25	..	25	23	33	27	25	43	37	34	35	46
Average increase <sup>1</sup>				..	- 4	+ 2	0	0	+ 3	+ 3	- 2	+ 1	+ 5

<sup>1</sup> These figures obtained by setting for each individual a standard score equal to the highest of his first three scores on the table, by calculating for each later score the difference from the standard, and then by averaging the individual differences for each week.

The blood chemical and hematological findings were completely negative. There was no trend toward a lowering of either albumin or total protein; the red blood count and hemoglobin remained essentially unchanged.<sup>3</sup>

In the case of this group the final control period had a special significance since the protein in the diet was raised to a high level at this time. No change in physical performance or chemical findings was evident aside from the increased amount of nitrogen in the urine. The subjective effect of the change was conditioned by the preferences of the men: two men, habitual lovers of meat, were much happier on the high protein diet; two men found the high protein diet actually distasteful, the remainder had no strong feelings one way or the other.

## *II. High protein group*

Again in this group the changes due to alterations in diet were practically non-existent. The weekly averages of daily protein intake ranged from 157 to 192 gm. compared to 95 to 113 in the normal group (see table 1 for 4-week averages). The average in the high protein group was kept down by one subject, a sedentary, rather hypersensitive individual to whom "heavy food" was distasteful.

The less active men in this group tended to feel overfull and sleepy after meals. Not all this effect was necessarily due to the high protein per se. Among the available common foods high protein is associated with considerable fat. As a result the total caloric intake in this group tended to be higher than necessary. This is clearly shown in the weight figures which show an average gain of 1.0 kg. and a gain in one individual of over 5 kg. No other unusual symptoms were experienced.

The physical fitness tests showed a tendency to gradual improvement (table 2) as in the other groups, which is likewise explainable by training.

The urinary excretion of nitrogen expressed as equivalent protein in table 1 gives figures of 138 gm. and 142 gm. daily for the two 4-week periods. Although not exactly to be compared with the intake figures because of the less frequent measurements (see under Restricted Protein Group above), these figures confirm the high protein metabolism of this group but would require the assumption of rather high values of stool nitrogen (about 6 gm.) to be brought in line with the calculated intakes.

<sup>3</sup> Details of these measurements are omitted for the sake of brevity but will be furnished by the authors on request.



The only change in chemical findings<sup>3</sup> was a tendency to slightly higher NPN which is probably not very significant.

#### DISCUSSION

These studies fail to confirm Chittenden's conclusions that a restricted protein diet improves physical well being. His experiments had the advantage of being longer in duration but the disadvantage of having no parallel control subjects on their usual diet. His conclusion that there is no impairment of health by such a diet is completely supported.

It is apparent from these studies that to choose a low protein diet from commercial articles of diet is difficult, provided caloric balance is maintained. When sufficient calories are given for hard physical labor, a fairly respectable level of protein is automatically included.

Likewise it was found difficult to increase the protein level above 150 gm. using standard items of diet. To push beyond this without giving excessive calories, defatted foods such as buttermilk or skim milk would be necessary.

The conclusions from this study should not be extended beyond the conditions investigated. The protein needs during growth, illness and lactation are wholly outside the scope of this work. It is apparent only that for 2 months with normal men (such as laborers or soldiers) rather extreme variations in protein intake were without measurable effect either beneficial or harmful. The practical implications are that under emergency conditions a diet supplying about 50 gm. of protein, chiefly from potatoes and grain products, is not incompatible with the health of physically active young men.

#### SUMMARY AND CONCLUSIONS

1. Within 2 months no measurable influence either deleterious or beneficial could be observed on the physical vigor or efficiency of eight healthy young men subsisting on a diet adequate in calories but restricted in protein. The daily protein intake averaged 50 to 55 gm., very little of which was of animal origin.

2. Similarly no beneficial or harmful effect could be observed in 2 months on eight men subsisting on a diet providing 160 gm. or more of protein, mostly first class.

## ACKNOWLEDGMENTS

This study was made possible only by the complete cooperation of the authorities and personnel of C. P. S. Camp no. 32 where it was held. The authorities concerned include both those of the Selective Service System and of the American Friends Service Committee. The camp personnel to whom we are indebted include not only the volunteer subjects, who added the hardships of the diet to a busy schedule, but also to various members of the maintenance personnel who assisted in many tasks connected with the experiments.

We are indebted to Dr. F. J. Stare and Dr. D. M. Hegsted of the Nutrition Department, Harvard Medical School for helpful suggestions on the choice and preparation of diets and for their loan of dietitians.

Various members of the U. S. Forestry Service assisted us throughout by various courtesies.

We acknowledge substantial financial assistance from the Nutrition Foundation, Inc.

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## ABSENCE OF NERVE DEGENERATION IN CHRONIC THIAMINE DEFICIENCY IN PIGS<sup>1</sup>

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### ONE FIGURE

(Received for publication April 18, 1944)

In earlier reports (Wintrobe, Stein, Miller, Follis, Najjar, and Humphreys, '42 b; Follis, Miller, Wintrobe and Stein, '43) we described our failure to produce degenerative changes in the nervous system of pigs by inducing thiamine deficiency. This observation was contrary to the rather generally held view that thiamine is "the anti-neuritic vitamin." Since our studies at that time were limited to nine pigs, this report concerning observations in ten additional animals is recorded. In the accompanying table the pertinent data for all nineteen animals are summarized.

The care and management of the animals was similar to that followed in earlier studies (Wintrobe, Miller, Follis, Stein, Mushatt and Humphreys, '42 a) and need not be described in detail again. The basal diet consisted of casein 26.1, sucrose 57.7, lard 11.0, and a complete salt mixture 5.2. In the present series there were four pigs which received only crystalline vitamins as the source of the "B-complex." Six animals were given autoclaved yeast. This was done because in most of the experiments reported hitherto, diets supplemented by autoclaved yeast have been used, the purpose of autoclaving being to destroy thiamine. Since we have observed neurological lesions in pigs receiving a diet deficient in pyridoxine or pantothenic acid (Wintrobe, Miller, Follis, Stein, Mushatt and Humphreys, '42 a), the question arose whether the pyridoxine or, more probably, the pantothenic acid content of yeast might also be lowered by autoclaving. Two of our animals receiving autoclaved yeast were given pyridoxine and calcium pantothenate as well.

<sup>1</sup> Aided by grants from the Rockefeller Foundation, Parke-Davis and Company, the Upjohn Company, and the Fleischmann Laboratories, and carried out in cooperation with the Bureau of Animal Industry, U. S. Department of Agriculture.

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The yeast used was a brewers' yeast available commercially.<sup>3</sup> "Dry" autoclaved yeast was prepared by autoclaving for 6 hours at 20 pounds pressure. "Alkaline" autoclaved yeast was prepared by mixing yeast with 0.1 N sodium hydroxide and autoclaving as above, neutralization being carried out afterwards. These two methods of autoclaving were employed because each method has been used by various workers (Swank, '40; Bessey, '42). The pantothenic acid content of the untreated yeast, as measured by microbiological assay (Strong, Feeney and Earle, '41) was 72  $\mu$ g. per gm. That of the "dry" autoclaved yeast was 14  $\mu$ g. while the "alkaline" autoclaved material contained 46  $\mu$ g.

In none of the animals was abnormal gait observed during life and in none were histologic changes discovered in the nervous system in spite of painstaking study by accepted methods. This is in agreement with the observations of Ellis and Madsen ('44). There can be no question that thiamine deficiency was produced in these animals for loss of appetite, vomiting and impairment of growth were observed in all, these symptoms could be relieved by giving thiamine, and death occurred as the result of cardiac failure in all except those animals which were sacrificed in a condition of less severe thiamine deficiency. Pronounced electrocardiographic changes were observed in these pigs (Wintrobe, Alcayaga, Humphreys and Follis, '43a) as well as extensive lesions in the myocardium and evidence of congestive cardiac failure (Follis, Miller, Wintrobe and Stein, '43).

In order to imitate the conditions likely to be encountered in humans, the thiamine deficiency was only partial. This procedure also achieved another purpose. It has been argued that nerve degeneration is not to be expected in animals acutely deficient in thiamine and it has been implied that changes in the nervous system will be observed if the deficiency is of sufficiently long standing. It will be noted that many of our animals were maintained in a chronically deficient state for 7 to 8½ months, one even longer. The growth curves shown in figure 1 and those shown in an earlier report (Wintrobe, Stein, Miller, Follis, Najjar and Humphreys, '42 b), indicate the "chronicity" of these experiments. Once the experiment was commenced, care was taken at all times to maintain the thiamine supplement at a level well below that required by the pigs. This level was so low at times that some of the pigs died sooner than we would have wished (see table 1).

<sup>3</sup> Mead Johnson and Company, Evansville, Ind.

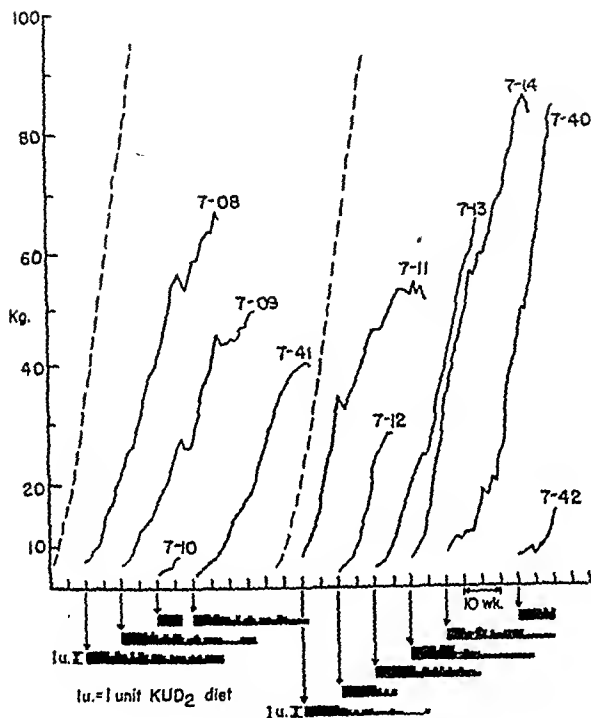


Fig. 1 Growth curves and food consumption of animals receiving thiamine-deficient diets. One unit of the diet furnished approximately 152 cal. per kilogram body weight.

The broken curve appearing at intervals represents the growth of pigs at the Beltsville Research Center given a mixed diet and fed and handled according to record of performance procedure.

#### DISCUSSION

It does not necessarily follow as a result of these studies that nerve degeneration in man is never the result of thiamine deficiency. There has been ample demonstration of the fact that the manifestations of vitamin deficiency, as well as the requirements for the various vitamins, are not the same in all species. It is to be noted, however, that when nerve degeneration has developed in man in association with nutritional deficiency, the deficiency has been multiple and there has

TABLE 1.

*Summary of data for 19 thiamine-deficient pigs.*

DIETARY SUPPLEMENTS	PIG NO.	AGE EXPT. ENDED	DURATION OF DEFICIENCY	AMOUNT OF THIAMINE GIVEN Average and range	CAUSE OF DEATH	NERVOUS SYSTEM
		days	days	μg./kg. body wt.		
RNB <sub>6</sub> ChP <sup>1</sup>	6-53 <sup>2</sup>	246	156	14 ( 0-35)	Cardiac failure	Normal
	6-67 <sup>2</sup>	171	83	0	Cardiac failure	Normal
	6-77 <sup>2</sup>	334	246	14 ( 0-35)	Cardiac failure	Normal
	6-90 <sup>2</sup>	134	57	10 (10)	Cardiac failure	Normal
	6-91 <sup>2</sup>	107	37	10 (10)	Cardiac failure	Normal
	6-92 <sup>2</sup>	210	141	20 (10-60)	Sacrificed	Normal
RNB <sub>6</sub> ChPIp	7-08	296	258	17 ( 3-65)	Cardiac failure	Normal
	7-09	290	254	15 ( 3-65)	Cardiac failure	Normal
	7-10	76	41	28 (15-65)	Cardiac failure	Normal
	7-41	238	202	17 (10-20)	Cardiac failure	Normal
Yeast, autoclaved (dry) <sup>3</sup>	7-11	273	231	0	Cardiac failure	Normal
Yeast, autoclaved (dry) <sup>3</sup>	7-40	244	206	0	Sacrificed	Normal
Yeast, auto. (dry) B <sub>6</sub> P <sup>1</sup>	7-14	280	238	0	Sacrificed	Normal
Yeast, autoclaved (alk.)	7-12	147	103	(See footnote 4)	Cardiac failure	Normal
Yeast, autoclaved (alk.)	7-42	101	67	(See footnote 4)	Cardiac failure	Normal
Yeast, auto. (alk.) B <sub>6</sub> P <sup>1</sup>	7-13	241	204	(See footnote 4)	Sacrificed	Normal
Liver, desiccated	6-54	410	320	42 ? (40-90)	Cardiac failure	Normal
	6-62	327	239	42 ? (40-60)	Sacrificed	Normal
	6-79	318	230	41 ? (40-50)	Sacrificed	Normal

<sup>1</sup> R refers to riboflavin 0.12, N niacin 1.20, B<sub>6</sub> pyridoxine 0.20, Ch choline 10.00, P calcium pantothenate 0.50, I inositol 0.10, p para-aminobenzoic acid 0.1 mg. per kilo body weight daily.

<sup>2</sup> Animals in previous series (Wintrobe et al., '42 b; Follis et al., '43).

<sup>3</sup> Pig 7-11 was fed alkaline autoclaved yeast until the age of 138 days. Pig 7-40 was given 0.50 mg. calcium pantothenate per kilo body weight daily until the age of 103 days. Thus both animals received a larger supply of calcium pantothenate than they would have had, had the yeast supplement from the age of 3 weeks been dry autoclaved yeast. All pigs receiving yeast were fed 3 gm. per kilo body weight daily.

<sup>4</sup> The thiamine content of these yeast samples was not measured.

been no adequate justification for the assumption that the lack of thiamine was the specific cause of the changes observed. Surely classical beriberi, occurring as it does in persons consuming a diet consisting almost exclusively of polished rice, is a multiple deficiency disorder. The effects of therapy with thiamine in cases of "multiple neuritis" have not been such as to convince the critical observer that eventual improvement must necessarily have been attributed to thiamine (Brown, '41; Strauss, '43).

The only exceptions to the above statement are the reports of Williams et al., ('43) and Najjar and Holt ('43). Williams and coworkers

described symptoms and signs suggesting peripheral nerve degeneration in two experimental subjects given a diet lacking only in thiamine. Unfortunately no biopsy was made and thus it was impossible to state whether there was functional impairment only, or actual nerve degeneration as well. The same question arises in the case of the four subjects of Holt and Najjar's study in which "neuritis" developed. In view of the importance of thiamine in carbohydrate metabolism one must also consider the possibility that some of the symptoms and signs regarded as representing "polynuropathy" may be the result of a metabolic disturbance in the muscles. Surely those signs which are promptly relieved by the administration of thiamine are the consequence of a metabolic defect rather than due to a histologic change.

It is of interest to compare our results with those of Van Etten, Ellis and Madsen ('40), also obtained with pigs. These workers observed neurological changes in animals fed autoclaved liver and whey as the source of the B vitamins, but found no such changes in pigs fed sodium sulfite, sulfur dioxide treated liver and whey. Autoclaving of liver for 6 hours at 20 pounds pressure, was found by our assays to reduce the pantothenic acid content from 105 to 34  $\mu\text{g.}$  per gm.; autoclaving of whey in the same way, reduced the pantothenic acid from 33 to 4  $\mu\text{g.}$  Incomplete data suggest that sulfite treatment of these substances as well as of yeast does not reduce their pantothenic acid content to the extent produced by autoclaving.

The requirement of the pig for pantothenic acid is at least 160  $\mu\text{g.}$  per kg. body weight (Wintrobe, Follis, Alcayaga, Paulson and Humphreys, '43 b), and under certain conditions may be as high as 500  $\mu\text{g.}$  (Ellis, Madsen and Miller, '43). The observations of Van Etten, Ellis and Madsen ('40) indicated that their autoclaved diet was deficient in more than one factor. In the light of our assays, pantothenic acid probably was one of these factors and the neurological changes observed by them were in all likelihood due, in part at least, to a lack of this vitamin. The absence of nerve degeneration in their animals which were fed sulfite treated diets, and in our pigs fed autoclaved yeast, can be explained by a failure to reduce the pantothenic acid content below the critical level.

#### SUMMARY

Additional experiments in pigs in which chronic thiamine deficiency was produced, have failed to support the claim that lack of thiamine causes degenerative changes in the nervous system.



TABLE 1

*Summary of data for 19 thiamine-deficient pigs.*

DIETARY SUPPLEMENTS	PIG NO.	AGE EXPT. ENDED	DURATION OF DEFICIENCY	AMOUNT OF THIAMINE GIVEN	CAUSE OF DEATH	NERVOUS SYSTEM
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The only exceptions to the above statement are the reports of Williams et al., ('43) and Najjar and Holt ('43). Williams and coworkers

# INEFFECTIVENESS OF VITAMIN E IN PREVENTING CHOLESTEROL DEPOSITION IN THE AORTA<sup>1</sup>

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The fact that vitamin E deficiency causes an increase in the cholesterol content of muscles in rabbits, rats and chicks (Morgulis, et al., '38; Heinrich and Mattill, '43; Dam, '44a) and affects at least part of the vascular system, i. e., the capillaries, in chicks (Dam and Glavind, '39) and in the rat's fetus (Mason, '42, '43) naturally leads to the question whether the presence or absence of vitamin E might not influence the deposition of cholesterol in the aorta of animals fed a diet rich in this substance.

An investigation of this question has been carried out with rabbits and chicks, species which are known to deposit cholesterol in the aorta during prolonged cholesterol-feeding. In the experiments with chicks the additional effect of orally administered lipoeic and inositol was studied, since these substances are known to exert an influence on the vascular symptoms in vitamin E deficient chicks (Dam and Glavind, '42); while lipoeic has also been reported by certain authors to counteract cholesterol atherosclerosis in rabbits, other investigators appear not to be in accord with this finding. The experiments with rabbits were carried out first. Since the aorta showed no significant changes it was decided in the chick experiments to extend the cholesterol determinations to certain other tissues in addition to the aorta.

## EXPERIMENTAL

In the experiments with rabbits 3 groups of young animals weighing about 1400 gm. were reared on a basal diet of ground oats to which was added 5 mg. diacetate of 2-methyl-1, 4 naphthohydroquinone per kg., supplemented with 50 gm. of carrots per animal per day. One group was given 1% powdered cholesterol mixed with the ground

<sup>1</sup> Aided by a grant from the Josiah Macy, Jr., Foundation.

oats<sup>2</sup> and another group received 100 mg. d,l-alpha-tocopherol acetate<sup>3</sup> per kg. of the oats-cholesterol mixture. After 85-97 days of feeding the animals were killed and the aortas, from the aortic valve to the bifurcation, were removed and analyzed for cholesterol after the method of Hoffmeyer and Björnsson ('40). This procedure involves hardening the tissue in 4% formaldehyde solution for 24 hours, removal of the adventitial fat by scraping, drying in vacuo at room temperature to constant weight, dissolution in 60% KOH by heating on a steam bath, extraction with ether, washing and evaporation to dryness. After dissolving the non-saponifiable matter in chloroform the cholesterol was determined in an aliquot part by the Liebermann-Burchard reaction. The results are presented in table 1.

Chicks were reared in ordinary brooders with wire screen bottom and fed, during the first 61 days of life, a basal diet deficient in vitamin E and containing 30% lard (diet 190, Dam, '44a). Supplements of cholesterol, tocopherol,<sup>3</sup> inositol and lipocaic<sup>4</sup> were given as indicated in table 2. The aortas of the chicks in each experimental group were pooled and analyzed for cholesterol by the same method used for rabbit aortas. In order to compare the possible effect on the aorta with that on other organs, livers, breast muscles and brains (cerebrum and cerebellum) were also examined, 1-gm. samples of these organs being dried in vacuo to constant weight without formalin treatment. For practical reasons it was necessary to pool the corresponding tissues from different individuals in some groups and therefore only average figures are presented (table 2). The experiments arranged between the horizontal lines in table 2 were carried out simultaneously.

## RESULTS

### *Rabbit experiments*

According to Hoffmeyer and Björnsson ('40) the upper limit for normal cholesterol values in rabbit aortas, as determined by their method, is 5 mg. per gm. dry weight. In the present experiment the group without ingested cholesterol showed values from 5.7 to 10.2 mg. per gm. The reason for this is not clear. The values for the aorta cholesterol in the two cholesterol-fed groups are irregular but increased values oc-

<sup>2</sup> Although cholesterol is usually fed as a solution in oil to facilitate its absorption, the relatively large amounts fed in the powdered form compensated for the lower absorbability and caused deposition in the aorta and other tissue.

<sup>3</sup> "Ephynal acetate," kindly supplied by Hoffmann-La Roche, Inc., Nutley, N. J.

<sup>4</sup> Kindly supplied by Dr. L. R. Dragstedt, University of Chicago and The Eli Lilly Research Laboratory.

cur not only in the group which received no added vitamin E but also in the group which received tocopherol. This latter substance, therefore, did not prevent the cholesterol deposition. It is of little importance for this conclusion that the basal diet was not devoid of vitamin E.

TABLE 1

*Influence of feeding cholesterol and vitamin E on mortality and cholesterol content of the aorta of rabbits.*

GROUP	ADDITION TO BASAL DIET	DAYS ON DIET UNTIL		INITIAL WEIGHT	FINAL WEIGHT	TOTAL CHOLESTEROL IN DRIED AORTA
		Death	Killed			
				gm	gm	mg per gm
		95		1430	927	5.7
		52		1550	1221	
			92	1347	1095	6.4
			91	1640	1498	6.5
1	none		92	1365	1623	9.5
			85	1355	1268	10.2
		37		1530	1122	
			92	1505	2175	9.7
		25		1425	940	
		50		1630	1144	
		93		1355	1476	10.6
		58		1422	1315	
		73		1375	1010	
2	1% cholesterol		92	1335	1456	15.5
		25		1388	1080	
		48		1270	990	
		45		1475	1020	
		41		1461	879	
			88	1421	1244	32.7
		56		1316	1115	
	1% cholesterol		97	1440	1380	5.1
			97	1345	1585	7.5
	+ 10 mg.% d, l-alpha	56		1345	955	
3	tocopherol		92	1392	1458	18.7
			92	1345	1228	22.0
	acetate		92	1390	1510	58.6

While the rabbits grew slowly and irregularly on the basal diet and some of them died, the cholesterol feeding without added vitamin E caused a considerable increase in the mortality, making it necessary to include many more individuals in group 2 in order to obtain aortas which had been exposed to the cholesterol feeding for the same length of time as in the other groups. Ingestion of a substantial amount of d, l-alpha-tocopherol acetate lowered the mortality. This seems to in-

TABLE 2

*Influence of cholesterol feeding with and without vitamin E, and with vitamin E and inositol or lipocaine, on the cholesterol content of the aorta and other organs in chicks during the first 61 days of life.*

GROUP	ADDITION TO BASAL DIET	NO. OF ANIMALS		WEIGHT WHEN KILLED GM.	AORTA DRY WT.	LIVER		CHOLESTEROL, AS MG. PER GM. OF TISSUE				BRAIN	
		Initial	Final			Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.	Wet wt.	Dry wt.
1	none	9	4 <sup>1</sup>	368	8.54	3.22	11.1	1.45	5.80	15.5	75.0		
2	10 mg. % d, l. alpha toco- pherol ace- tate	8	8	422	9.43	3.55	11.2	0.88	3.35	15.9	74.2		
3	2% cholest- erol	10	6 <sup>2</sup>	390	27.5	50.9	151	1.95	7.96	15.9	75.2		
4	as 3 + 10 mg. % d, l-alpha tocopherol acetate	10	10	365	28.6	46.9	138.5	1.04	4.10	15.0	72.8		
5	same as 4	7	7	424	30.8	48.8	146	1.01	3.88				
6	as 4 + 1.5% inositol	7	6	344	29.7	30.8	95.2	1.19	4.64				
7	as 4 + 2% lipocaine	7	7	532	32.2	88.0	234	0.966	3.63				

<sup>1</sup> Four of the animals which died during the feeding had encephalomalacia and one had general exudative diathesis. Of the four surviving birds used for the determinations, two had a general exudative diathesis; and none had encephalomalacia.

<sup>2</sup> Three of the animals which died during the feeding had general exudative diathesis, none had encephalomalacia (2% cholesterol protects largely against the latter symptom). Four of the surviving birds had general exudative diathesis; none had encephalomalacia.

dicating that the basal diet did not furnish vitamin E in sufficient amount to enable the animals to resist a general deleterious effect of the cholesterol feeding. The life-prolonging effect of vitamin E in these experiments in which the basal diet is unbalanced, probably also as far as the protein is concerned, resembles the effect of this vitamin on rats reared on a diet with fatally low protein content (Dam, '44b).

### *The chick experiment*

The chicks which were fed 2% cholesterol in addition to the high-fat diet had about three times as much cholesterol in the aorta as those which received the high-fat diet only. This finding that cholesterol-feeding causes deposition in the aorta in chicks is in accordance with those of Dauher and Katz ('42). The extent of cholesterol deposition was not significantly influenced by dietary supplements of vitamin E, or by additions of lipocaine or a large amount of inositol together with vitamin E. Since the diets contained yeast the experiments do not answer the question whether the cholesterol deposition would have been more marked if the basal diet had been completely free from inositol, but this circumstance does not interfere with the conclusion that vitamin E could not prevent the increase in aorta cholesterol even when lipocaine or inositol was added.

As already indicated, some workers (Huber et al., '37) have reported that lipocaine tends to inhibit deposition of cholesterol in the rabbit aorta while others (Vermeulen et al., '42) have been unable to confirm this. The present experiments, though carried out with another species, agree with the findings of Vermeulen et al., '42.

The data in table 2 show further that, just as was the case with respect to the aorta, the total cholesterol content of liver and brain was not influenced to any marked degree by the presence or absence of vitamin E, whereas the muscles showed a clear response. Additions of vitamin E to the basal diet, and to the basal diet with 2% cholesterol added, caused a decrease in muscle cholesterol somewhat greater than the increased deposition caused by the addition of cholesterol.

As was to be expected, the brain cholesterol did not change by cholesterol feeding whereas the liver took up a large quantity. The latter phenomenon was influenced in opposite directions by lipocaine and inositol. The finding that inositol reduced the cholesterol deposition in the liver is in agreement with the observations of Forbes ('43) but the finding that lipocaine increased it was surprising in view of what had been found in experiments with rabbits by Vermeulen et al. ('42).

Ingestion of high cholesterol together with high inositol seemed to have a noxious influence on the chicks since they had a mild perosis, were less vigorous and gained in weight more slowly than those receiving lipocaic or no addition to their cholesterol-rich diet. Ingestion of lipocaic together with cholesterol seemed to have an opposite effect. A more detailed study of the problems raised by these observations may throw some light on the effect of inositol and lipocaic on cholesterol metabolism.

#### SUMMARY

1. Vitamin E, fed as 10 mg. % d, l-alpha-tocopherol acetate in the basal diet, failed to modify the deposition of cholesterol in the aorta in (a) rabbits fed a diet of ground oats and carrots plus 1% cholesterol, and in (b) chicks fed an artificial diet deficient in vitamin E and containing 30% lard and 2% cholesterol, with or without addition of 1.5% inositol or 2% lipocaic.

2. The vitamin E supplement prevented a high mortality occurring in rabbits fed the oats-carrots-cholesterol diet, reduced the normal cholesterol content of muscles in chicks fed the basal artificial diet, and the increased muscle cholesterol of chicks fed the same diet supplemented with 2% cholesterol; the explanation of these effects is obscure.

3. In chicks inositol feeding reduced, and lipocaic feeding increased, the extensive deposition of liver cholesterol following additions of the latter to the basal diet.

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# TOXICITY OF FRACTIONS OF HOG LIVER FATTY ACIDS TO CHICKS FED A VITAMIN E DEFICIENT DIET<sup>1</sup>

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TWO FIGURES

(Received for publication May 16, 1944)

It is known that chicks reared on a vitamin E deficient diet will develop encephalomalacia or exudative diathesis when the diet contains a sufficient amount of fat containing highly unsaturated fatty acids. Which of the two symptoms will be the main clinical manifestation of the disorder is largely determined by the composition of the diet (Dam, '44). The nature and amount of the fatty acids in the diet is particularly influential in this respect. For instance, with the basal diet 182 (Dam, '44) the main symptom will be exudative diathesis when the fat supplement of the diet (about 5%) consists of the fatty acid mixture from cod liver oil, but encephalomalacia when it consists of the fatty acids from hog liver fat.

The various highly unsaturated fatty acids are, at present, not easily available in sufficient quantities for a comparison of their ability to provoke these two symptoms. It has therefore been attempted, as a preliminary step in this investigation, to divide the fatty acids from hog liver fat into three fractions of varying degree of unsaturation and to compare the effect of these fractions on the symptoms.

## EXPERIMENTAL

Seven and one-half kilograms of powdered dried hog liver were extracted with ether in a continuous extractor for 24 hours. The fat was saponified at room temperature in ether solution with 100% excess of potassium hydroxide in methanol for 24 hours, after which water was added and the non-saponifiable fraction removed by many shakings with ether. After acidification with HCl, the fatty acids were extracted with ether, washed with water, dried with sodium sulfate and the ether removed in vacuo. The fractionation of the fatty acids was carried out

<sup>1</sup> Aided by a grant from the Josiah Macy, Jr., Foundation.

in accordance with the method of Brown and Stoner ('37); namely, by preparing a 10% solution in acetone, freezing at three different temperatures, filtering and washing. The following fractions were obtained: fraction 1, separated from the acetone solution above  $-20^{\circ}\text{C}$ ., solid at room temperature, iodine value 5, amount 241 gm.; fraction 2, separated between  $-20^{\circ}\text{C}$ . and  $-72^{\circ}\text{C}$ ., liquid at room temperature, solid at  $0^{\circ}\text{C}$ ., iodine value 104, amount 216 gm.; fraction 3, remaining in solution at  $-72^{\circ}\text{C}$ ., liquid at  $0^{\circ}\text{C}$ ., iodine value 242, amount 200 gm.

The three fractions were tested with groups of ten newly hatched chicks as described by Dam ('44), first as 4% supplements to the vitamin E-free diet 182 (without cod liver oil). No other fat was given other than 1 drop of oleic acid (29 mg.) per animal twice a week as solvent for the concentrates of vitamins A and D. The fractions were added fresh to the diet every day, the bulk of them being kept in vacuo at  $0^{\circ}\text{C}$ . during the period of the experiment. The peroxide value of the most unsaturated fraction, determined after the method of King et al. ('33), was less than 4 milliequivalents per 100 gm. at the end of the experiment and the iodine was unchanged. In the case of fraction 3 which proved the most active one, the protective action of d, l-alpha-tocopherol acetate was tested, and also the effect of a much smaller amount of this fraction was determined, namely, 1 drop (24 mg.) per animal per day during the first 10 days, the daily dose being increased by 1 drop per animal for every subsequent 10-day period. The amount given in this way corresponded to approximately 0.5-0.6% of the diet consumed.

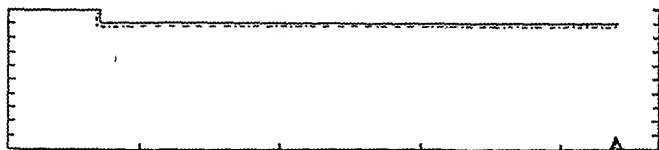
The method of observing and recording the symptoms was as previously described (Dam, '44). The graphs give a survey of the results.

## RESULTS

The least unsaturated fraction 1 (exp. 2) gave only two instances of exudate, both fully developed but appearing rather late, and no encephalomalacia. Since neither oleic acid (Dam, '43, '44), palmitic, nor stearic acids (unpublished findings) give rise to symptoms when fed in similar amounts, the exudates must in this case be attributed to the small amount of highly unsaturated acids which accompanied the saturated acids in this fraction.

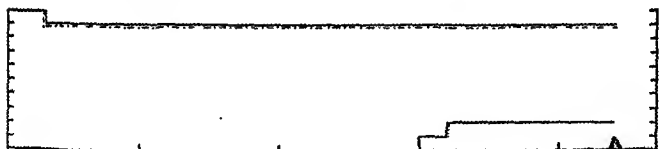
Fraction 2, consisting mainly of oleic acid together with small amounts of more highly unsaturated acids such as linoleic, produced both exudates and encephalomalacia with about the same frequency (exp. 3), the exudates appearing somewhat earlier than in experiment 2. One bird had an intrapericardial exudate, as previously observed (Dam, '44) in experiments with hog liver fatty acids.

Fraction 3, consisting mainly of linoleic and more unsaturated acids, gave a very early onset of severe encephalomalacia which killed nearly all the animals between the tenth and the twenty-first days of the experiment (exp. 4). At autopsy some of the birds in this group showed redness (fine hemorrhage) of the subcutaneous adipose tissue, which is the first stage in the development of exudate and is arbitrarily counted as a "half exudate" in the graph. The deleterious effect of

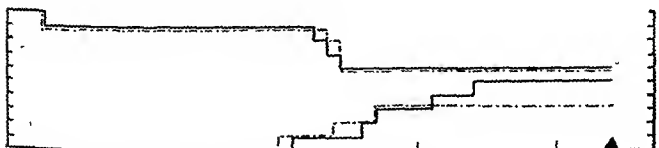


< 10 Days >

Exp.1. Diet 182 without cod liver oil



Exp.2. Diet as in Exp. 1 + 4% of fraction 1



Exp.3. Diet as in Exp. 1 + 4% of fraction 2

Abscissa: days.

Ordinate: number of animals.

Full line from bottom: development of exudate.

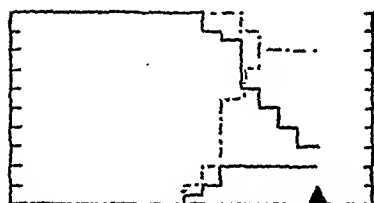
Full line from top: deaths of animals without exudate.

Broken line from bottom: development of encephalomalacia.

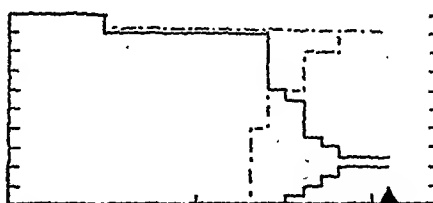
Broken line from top: deaths of animals without encephalomalacia.

fraction 3 was completely prevented when the diet contained 10 mg. % d, 1-alpha-tocopherol acetate<sup>2</sup> (exp. 5).

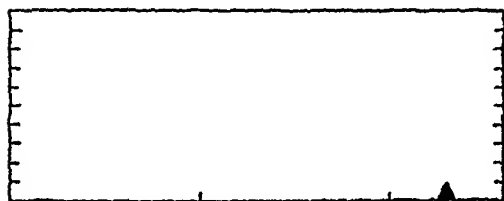
When fraction 3 was given in relatively small amounts corresponding to 0.5-0.6% of the diet (exp. 6), both encephalomalacia and fully de-



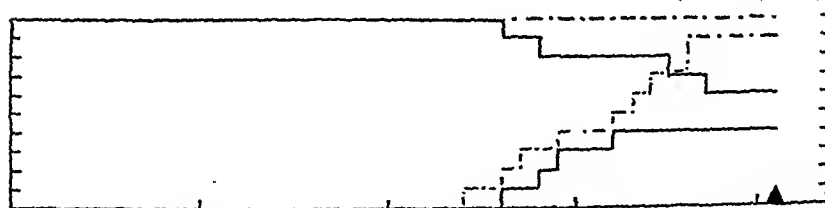
Exp. 4 Diet as in Exp. 1 + 4% of fraction 3



< 10 Days >  
Repétition of Exp. 4



Exp. 5 Diet as in Exp. 4 + 10 mg. % d, 1-alpha-tocopherol acetate.



Exp. 6. Diet as in Exp. 1 + "small amount" of fraction 3 (compare text)

Abscissa: days.

Ordinate: number of animals.

Full line from bottom: development of exudate.

Full line from top: deaths of animals without exudate.

Broken line from bottom: development of encephalomalacia.

Broken line from top: deaths of animals without encephalomalacia.

<sup>2</sup> Ten mg. % is probably far above the limit of the protective dose. This amount was chosen because it has been used with many other diets and always gave full protection. With diet 18<sup>2</sup> (containing 5% cod liver oil) the limit for protection has been found to lie between 0.625 and 1.25 mg. % in a 5-week experiment.

veloped exudates occurred, but these symptoms appeared more slowly than when the fraction was fed as 4% of the diet. Encephalomalacia was still the predominant symptom though less so than when 4% was given. None of the fractions tested gave encephalomalacia as the only symptom. It would nevertheless be of interest to study the effect of the individually fatty acids, especially those occurring in fraction 3.

#### DISCUSSION

The following represents a tentative interpretation of the observations reported here. As shown in previous experiments (Dam, '44) the time which elapses from the beginning of feeding to the onset of symptoms is sufficient for the accumulation of dietary fatty acids in the tissue lipids to such an extent that it influences the iodine value of the phospholipids, those of brain tissue being less altered than those of muscle and liver. It seems likely, therefore, that the presence of a certain amount of highly unsaturated fatty acids in the tissue lipids (phospholipids or others) is a prerequisite to the development of the symptoms. Since the two symptoms are both dependent upon ingestion of highly unsaturated fatty acids and preferably affect tissue which is rich in lipids (adipose tissue, brain tissue), and since the first gross change in the tissues in both cases consists of fine hemorrhages followed by edema or exudation, exudative diathesis and encephalomalacia may be due to the same biochemical disturbance occurring in different tissues — a possibility which has been referred to earlier (Dam, '44). It is known that the lipids of brain tissue are influenced less by the dietary fat than is the fat of adipose tissue, and it is therefore possible that ingestion of a small amount of unsaturated acids will mainly affect the adipose tissue or muscle tissue and cause exudative diathesis in this tissue when given sufficient time (exp. 2). However, if the brain tissue is more sensitive to a certain change of the lipids, the ingestion of a large amount of unsaturated acids may cause severe cerebellar or cerebral disturbances leading to death before the other symptom is fully developed (exp. 4).

The way in which the cellular damage is brought about could be one of the following: (1) The damage is caused by some abnormal oxidation of the highly unsaturated fatty acids in the tissue which can be prevented by the presence of a sufficient quantity of vitamin E, and can, locally, cause the destruction of the vitamin if the latter is present in such minute quantity that it cannot halt the oxidation, or (2) the presence of highly unsaturated fatty acids imparts such physical or physico-chemical properties to the lipid phase of the vascular wall, that

the latter, latently damaged by the absence of vitamin E, is more apt to give way to hydrostatic and osmotic forces. The possibility that destruction of traces of vitamin E in the diet through rancidity might play a role in the development of the symptoms has already been ruled out (Dam, '44).

The above-mentioned considerations do not explain the previously observed modifying effect of the protein-carbohydrate ratio, lipocae, inositol or cholesterol; neither do they explain the quantitative difference in the effect of hog liver fat and cod liver oil on the two symptoms.

#### SUMMARY

Three fractions of hog liver fatty acids were tested for their effectiveness in producing exudative diathesis and encephalomalacia in chicks reared on a vitamin E deficient diet.

When fed as 4% supplements to the diet, the least unsaturated fraction (iodine value 5) had little effect and gave only exudates; the intermediate fraction (iodine value 104) gave both exudates and encephalomalacia, whereas the most unsaturated fraction (iodine value 241) caused a rapid onset of severe encephalomalacia and early death of the animals. The deleterious effect of this fraction could be prevented by d, l-alpha-tocopherol acetate. When fed in smaller amounts corresponding to 0.5-0.6% of the diet, the most unsaturated fraction gave both exudates and encephalomalacia.

The significance of the incorporation of dietary fatty acids in tissue lipids as a prerequisite to the symptoms is discussed.

#### ACKNOWLEDGMENT

Thanks are due to F. Hoffmann-La Roche, Inc., Nutley, N. J., for d, l-alpha-tocopherol acetate (Ephynal acetate, Roche) and to Armour and Company, Chicago, for powdered dried hog liver.

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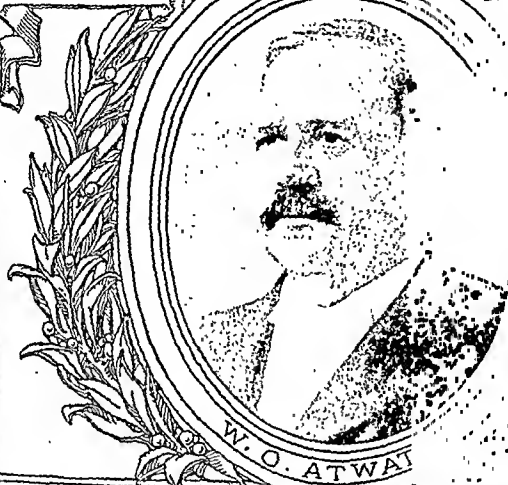
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# THE NUTRITIONAL SIGNIFICANCE OF INOSITOL

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*Laboratories of the Rockefeller Institute for Medical Research, New York*

(Received for publication June 29, 1944)

During the past few years interest in the nutritional properties of inositol has increased to such an extent that a review of published work in this field has seemed advisable, not only to survey and evaluate what has already been done, but also to delineate some of the paths that future investigators may find profitable. Limitations of space will not permit an exhaustive review of the subject of inositol, and hence the rôle which this substance plays in intermediary metabolism as well as some physiological and chemical considerations of interest must be passed over. An attempt will be made, however, to summarize briefly present knowledge of the requirements of various species for inositol in the diet.

## INOSITOL AS A MICROBIAL GROWTH FACTOR

The earliest observation on the nutritional significance of inositol was that of Eastcott (1) who isolated a growth stimulant for yeast from tea, and showed that it was meso-inositol. Eastcott's observation did not at first find ready acceptance (2), possibly due to inadequacies in biotin and pantothenic acid (2, 3) of some of the basal media used, but now has been amply confirmed and is used as the basis of the quantitative method for the estimation of inositol (4).

Several other fungi have been shown to require inositol for maximal growth (5, 6, 7), but thus far it has not been demonstrated to be required by any bacterial species. Indeed, many bacteria do not contain the substance in detectable amounts (8, 9), although certain others, notably *Pseudomonas* and several sporeforming species, contain considerable quantities. If this failure to detect inositol in several bacteria really is due to its absence from these forms and does not merely represent lack of sufficient sensitivity of the assay procedure, it constitutes an exception to the theory now current that all those water-soluble vitamins which are not required in the medium are synthesized by the cell.

## INOSITOL AS A DIETARY ESSENTIAL FOR ANIMALS

*Mice*

The present interest in the nutritional significance of inositol stems from the work of Woolley who in 1940 (10, 11) showed that a disease of mice of dietary origin, characterized by marked alopecia and retarded growth, was cured by inositol. A striking sign of the disease was the pattern of the alopecia (12). Hair was never lost from the head or tail or from the legs below the knees, while the areas of loss of hair on most other parts of the body were bilaterally symmetrical and in these areas the alopecia was nearly complete. In contrast to these findings Martin (13) observed only slight alopecia in mice fed a diet deficient in inositol. Woolley (9, 14) observed that the incidence of signs of inositol deficiency was usually not above 50% and that the amount of pantothenic acid in the diet had a marked influence on the appearance of the condition. Thus, in the absence of pantothenic acid, alopecia developed even though the ration contained adequate inositol. However, in the presence of large amounts of pantothenic acid and in the absence of inositol some animals still developed signs of inositol deficiency, and died unless the latter compound was administered. Analyses of the mice for inositol showed that the level fell to about half the normal value in the carcasses of affected animals (9).

While not of importance nutritionally, the work of Laszlo and Leuchtenberger (15) is of interest. These investigators reported that the daily intravenous injection of minute amounts of inositol prevented the development of transplanted tumors in mice. Strangely enough the effect was only observed following intravenous administration and was not seen when other routes were employed.

*Rats*

Shortly after the demonstration of the effect of inositol on alopecia in mice the influence of this substance on various experimental diseases of rats was examined. Gavin et al. (16, 17) reported that a type of fatty liver of rats induced by the feeding of biotin concentrates was treated successfully with inositol. Since that time several other investigators (18, 19) have observed beneficial effects of inositol on fatty livers in rats even when the fatty livers were produced without the aid of biotin. A lipotropic action of inositol in man under certain conditions has been reported (20). Pavcek and Baum (21) stated that the alopecia around the eyes seen in certain deficient animals (the so-called "spectacle eye") was promptly cured with inositol. Subsequently Nielsen and Elvehjem

(22) were inclined to view "spectacle eye" as a specific sign of biotin deficiency not curable by inositol.

There are data which indicate that the rate of growth of rats fed an otherwise adequate, highly purified diet is increased by inositol (18). On the other hand, rats have been raised to maturity on a diet free of inositol (23, 24). Martin (25) reported that the addition of p-aminobenzoic acid to a highly purified ration produced a syndrome characterized by poor growth and changes in fur which was preventable by inositol. Somewhat analogous effects have been observed by others (26, 27). Curiously enough, the diet when supplemented with neither p-aminobenzoic acid nor inositol was adequate for the rats. Furthermore when this ration was supplemented with inositol alone a definite deleterious effect was observed which was prevented by p-aminobenzoic acid.

Sure (28) presented data which indicated that inositol and p-aminobenzoic acid, especially the former, when fed to mothers receiving a highly purified diet, considerably improved the percentage survival of newborn rats. In a subsequent paper (27) Sure attributed the action largely to p-aminobenzoic acid. Climenko and McChesney (29), on the other hand, attributed the action largely to inositol.

When diets of more complex nature than the ones in the above studies were used, a striking, beneficial effect of inositol on rats was observed by various workers. Thus Cunha et al. (30) found that when rats were fed a diet made up largely of corn, soybean meal, salts, and alfalfa, they grew suboptimally and developed alopecia, similar in distribution and character to that observed by Woolley in inositol-deficient mice. That the syndrome represented inositol deficiency in rats was readily demonstrated by feeding this compound. Shortly after the appearance of this paper Nielsen and Black (31) showed that when rats were fed a highly purified diet containing all of the known vitamins including biotin and folic acid, and sulfasuccidine, they failed to grow optimally and developed alopecia. Growth was restored and alopecia prevented by administration of inositol. The sulfonamide had in some way brought out a need for inositol.

#### *Cotton rats*

McIntire et al. (32) reported that the addition of inositol to a highly purified diet practically doubled the rate of growth of cotton rats. This is the greatest effect on growth rate which has been observed with any species in which highly purified diets supplied with ample amounts of the known vitamins (and not supplemented with sulfonamides) were used.



*Guinea pigs*

Hogan and Hamilton (33) observed that the rate of growth of guinea pigs fed on a partially purified diet was increased by the addition of inositol.

*Hamsters*

Hamilton and Hogan (34) reported that, while the rate of growth of hamsters was not increased by inositol, those animals raised on the inositol-deficient diet had difficulty in reproduction. Many of the young were born dead or as bloody shapeless masses, and some of the mothers succumbed during parturition. Animals fed inositol did not show these signs. Cooperman et al. (35) observed increased rate of growth in hamsters following addition of inositol to a purified diet.

*Chickens*

Hegsted et al. (36) reported that the rate of growth of chicks fed a partially purified diet was increased slightly by addition of inositol. Dam (37) observed that inositol added to a tocopherol-deficient diet prevented encephalomalacia and exudative diathesis — two signs frequently seen in tocopherol-deficient chicks. In this connection it is interesting to speculate whether the substance in soybean oil reported to be necessary in addition to tocopherol for the cure of tocopherol deficiency in chicks (38), may not be lipositol (39) the inositol-containing phosphatide which is present in large amounts in soybeans.<sup>1</sup>

## SYNTHESIS OF INOSITOL BY ANIMALS

In considering the nutritional significance of inositol it is necessary to note that several species of animals have been shown to synthesize the compound. Vohl in 1858 (40) was able to isolate quantities of inositol from the urine of a man with diabetes insipidus far in excess of the amount we now know could have been obtained from the food. The extra inositol excreted probably arose from synthesis, or possibly from tissue breakdown. Following this lead, Needham in 1924 (41) showed that rats rendered polyuric by administration of salt excreted more inositol than was ingested. Since the excretion of this excess of inositol continued over long periods, it was concluded that the rats were synthesizing the substance. Woolley (9) demonstrated that mice fed a synthetic diet frequently synthesized inositol in amounts almost equal to the minimal effective dose of the compound for this species. At least one point of origin of this synthesized inositol was shown to be the in-

<sup>1</sup> Patrick has recently (Gibson Island Conference, 1944) recognized that this effect is merely due to the antioxidant action of various phosphatides.

testinal flora of the mice. Needham (42) and Snell and Quarles (43) reported that during the incubation of eggs considerable inositol was synthesized. However, Woolley (44) showed that these observations on eggs were in error due to inadequacies of the methods of analysis used, and that what actually occurred during incubation was the conversion of an inositol compound into free inositol.

#### IS INOSITOL A DIETARY ESSENTIAL?

It is quite evident that there is no unanimity of opinion as to whether or not inositol should be regarded as a dietary essential. The fact that several workers have been able to obtain good growth of rats, mice, and chicks when these animals were fed highly purified diets very low in or devoid of inositol, and the fact that some of these species have been shown to synthesize inositol, must be pitted against the fact that several groups of workers have been able to observe moderate or marked beneficial results from the addition of inositol to the diets of mice, rats, guinea pigs, hamsters, cotton rats, and chicks. Several, but not all, of the less well marked responses and all of the failures have been observed in animals fed highly purified diets wherein it is customary to include amounts of the pure, water-soluble vitamins considerably in excess of the levels of these which occur in natural diets. Furthermore, there may be substances in natural foods which, although not themselves necessary to the species under investigation, nevertheless exert a marked influence on the need for exogenous inositol. Thus, Gavin and McHenry (16) showed that the addition of biotin to purified diets for rats caused the production of a disease (fatty livers) which was due to a dietary deficiency of inositol. Woolley (9, 14) showed that the incidence of alopecia as a sign of inositol deficiency in mice was in some measure dependent on the amount of pantothenic acid fed. In this connection, too, it is of interest to recall that Cunha et al. (30) observed their clear-cut case of inositol deficiency in rats not on a highly purified diet, but rather on one compounded from natural foodstuffs. Finally, in connection with the question of synthesis of inositol, it must be recalled that rats are able to synthesize choline (45) even though it can be demonstrated that they require choline or other suitable source of labile methyl groups in the diet.

The production of inositol deficiency in rats fed a highly purified diet plus sulfonamide drugs (31) indicated either that these drugs increased the demands of the organism beyond its synthetic capacities or that the intestinal microorganisms which were checked by the drugs were acting as a source of inositol. The demonstration in mice that in-

ositol-synthesizing microorganisms were present in the intestinal tracts of animals which did not exhibit signs of deficiency, or of those which cured spontaneously, but were not detectable in animals with signs of the deficiency (9) may lend support to the hypothesis that failure to observe inositol deficiency is due to the presence in the intestinal tract of bacteria which synthesize the compound. However, much more work will have to be done before this postulate can be regarded as established.

There are, finally, the several instances in which different groups of investigators have noted rather marked beneficial effects of inositol on several species of animals fed on highly purified diets. In some of these cases death resulted unless inositol was present in the diet. These observations were referred to in the preceding sections.

#### METHODS OF ANALYSIS

The most satisfactory method for the analysis of inositol now available is that which depends on the fact that inositol is a stimulant of the growth of *Saccharomyces cerevisiae* (4). It is specific, rapid, relatively accurate, and allows the separate estimation of both free and total inositol (9). Furthermore, it has the very considerable advantage that since it requires only minute amounts of inositol, very small samples of tissue suffice for a determination. Williams et al. (46) proposed a somewhat similar method, but it suffers from the fact that enzymic hydrolysis of the sample is employed to free combined inositol. One of the forms in which inositol occurs in tissues, namely lipositol (39), is not readily attacked by enzymes.

Several chemical methods have been advocated for the estimation of inositol. The combustion method of Needham (47) lacks specificity and does not determine some of the combined forms. Young (48) proposed a method based on the reaction of inositol with potassium iodo-mercurate, but the procedure is lengthy, requires large samples of tissue, and may lack specificity since many polyhydroxy compounds react with the reagent. Platt and Ghock (49) have advocated a method based on the fact that inositol, like other glycols, reacts with periodate. Steps were introduced to eliminate other naturally occurring glycols from the tissue extract before analysis. The results on comparable animal tissues were in reasonable agreement with those reported from the use of the yeast-growth method.

#### STRUCTURAL SPECIFICITY OF INOSITOL ACTION

The only compound which has yet been found to have appreciable inositol action for the growth of *Saccharomyces cerevisiae* is meso-

inositol (3). Some slight activity was shown by mytilitol, a methyl inositol obtained from mussels (50), and for inositol monophosphate. d-Inositol, l-inositol, the monomethyl ethers of these compounds, i. e., pinitol and quebrachitol, quereitol, quinic acid, inositol hexaacetate, phytin, inositol tetrphosphate, soybean lipositol, and inosose were inactive for the yeast. In mice, the results were similar except that the esters of inositol, namely, phytin, inositol hexaacetate, and soybean lipositol were active. These facts are important because most of the above compounds occur naturally (see below). It is therefore necessary to know which of them are of importance nutritionally. Also, since the esters of inositol are active for the animal but not for yeast, it is essential to convert these esters to the free compound before proceeding to measure the inositol content of foods by the yeast-growth method if a true evaluation of the foods is to be had.

It must be inferred from the results of Cunha et al. (30) that the inositol of phytin is not available to the rat. The ration which they used must have contained considerable phytin. If this inference is correct, the rat differs from the mouse in this respect.

#### CHEMICAL STRUCTURE OF INOSITOL

For a substance which has been under investigation for as long as inositol, it is rather surprising to realize that its complete chemical structure has been established only within the last 2 years. The reader will infer that there were special problems involved in order that this should be so. It is beyond the scope of this review to go into the steps by which the structure was finally established, but reference must be made to the work of Posternak (51) and of Dangschat and Fischer (52) who showed that the 6 hydroxyl groups are arranged about the cyclohexane ring in such a manner that four are below the plane of the ring and two are above. The two hydroxyls that are above the ring are in 1, 3 position.

#### NATURAL OCCURRENCE OF INOSITOL AND ITS DERIVATIVES

A number of isomers and analogs of inositol occur naturally, but since they do not exhibit inositol action nutritionally, only their existence will be noted here, and the reader will be referred to various handbooks such as that of Klein (53) for more extensive information.

Meso-inositol itself occurs in nature in at least four forms: free inositol, phytin, lipositol, and a water soluble, non-dialyzable complex. Free inositol has been isolated from both plant and animal sources. Phytin, the calcium and magnesium salt of inositol hexaphosphate, was

thought of until recently as an exclusive plant constituent. Its presence in many seeds has been known for decades. However, Rapoport (54) has shown that the erythrocytes of species such as the chicken and turtle in which these cells are nucleated contain appreciable quantities of phytin.

Inositol as a constituent of special phosphatides has been known from the work of Anderson (55) who isolated it from the phosphatides of the tubercle bacillus, and from the work of Klenk and Sakai (56) who obtained inositol monophosphate from soybean phosphatides. The widespread occurrence of inositol in phosphatides, however, was seen when Folch and Woolley (57) showed that it was combined in the cephalin fraction of brain and spinal cord and thus recognized a new inositol-containing phosphatide in animal tissues. Although the pure phosphatide has not yet been obtained from animal sources, a similar compound has been isolated from soybeans (39) and shown to be composed of inositol monophosphate in glycosidic linkage with galactose, and combined with ethanolamine, tartaric acid, oleic acid, and saturated fatty acids. It was named lipositol.

The occurrence of inositol as an integral part of a phosphatide and the rôle of inositol in the prevention of fatty livers bring to mind the occurrence of choline in a phosphatide and its vitamin-like action in prevention of fatty livers of a different character. Perhaps the formation of lipositol is one of the uses to which dietary inositol is put by the animal.

The existence of an inositol complex in heart muscle has seemed probable from the early observations of Rosenberger (58), but it was not until Winter's work (59) that this complex was demonstrated by controlled experiments. During autolysis of heart muscle of certain species the complex was broken down to free inositol. It does not seem probable that this complex in heart muscle is lipositol since that organ is low in this compound, and since lipositol is not readily hydrolyzed during autolysis of tissues. Woolley (11) recognized a water-soluble, non-dialyzable inositol complex in liver, and showed (9) that inositol complexes were present in most of the tissues that were examined.

Most plant and animal tissues thus far examined have been found to contain between 0.05 and 0.5% of total inositol on a dry weight basis (4, 60). Heart was the richest natural source since 1.6% of the dry weight of this organ was found as inositol. In the few tissues which have been analyzed, approximately one-half of the total inositol was free (9), although the proportion which was uncombined varied from tissue

to tissue. The amount of total inositol in many natural diets is of the same order of magnitude as that which has been found to prevent signs of inositol deficiency in the various species examined.

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# THE EFFECTS OF SUGARS ON THE RESPIRATORY EXCHANGE OF CATS<sup>1</sup>

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In our general program of research on the variations in the respiratory quotient and in the total metabolism of different species of animals after the ingestion of sugars, the cat has been studied as a representative of the Carnivora. Usually the diet of the cat is mainly of animal origin, although domesticated cats can be trained to eat foods of plant origin.

## METHODS AND PROCEDURE

The respiratory exchange of five nearly adult or fully grown cats was determined by the open-circuit method, proportionate samples of the ventilating air current being collected in the Fox bag (Carpenter and Fox, '31a) and subsequently analyzed by means of a gas-analysis apparatus (Carpenter, '33). The gas-analysis apparatus was standardized daily by analyses of samples either of outdoor air or of air in which ethyl alcohol was burning. The average respiratory quotient of 129 samples of air passing over burning alcohol was 0.665 with a standard deviation of  $\pm 0.0059$ .

The respiration experiments<sup>2</sup> were made from 16 to 24 hours after food had been removed from the cats. The room temperature at which the cats were kept prior to the experiments varied according to the season, but the measurements of respiratory exchange were made at environmental temperatures between 20° and 28°C., usually 22° to 26°C. Observations were made during eight successive ½-hour periods under basal conditions (no dose), after ingestion of 75 ml. of water as a control, and after ingestion of 10 gm. of glucose, fructose, galactose, lactose, maltose, or sucrose, and combinations of 5 gm. each of glucose and galactose or of glucose and fructose. The sugars were dissolved in 50 ml. of water at 37°C. and given by stomach tube, and 25

<sup>1</sup> A preliminary report has been presented in the Federation Proceedings, vol. 3, p. 93 ('44).

<sup>2</sup> The respiration experiments were carried out with the technical assistance of Mr. Basil James.



ml. of water was used to rinse the funnel and the tube. The measurements of the respiratory exchange were usually begun about 20 minutes after the feeding.

All the cats were male except A. Cat G was old, not in good physical condition, and finally died of some pulmonary difficulty.

In the comparisons made in the following pages between cats and man, the statements regarding man are based on reports previously published by Carpenter and Fox ('30a, '30b, '31b, '31c), Carpenter and Lee ('32a, '32b, '37a, '37b, '37c, '38), and Root and Carpenter ('42).

## RESULTS

### *Respiratory quotient (R. Q.)*

The average respiratory quotients in the successive  $\frac{1}{2}$ -hour periods of measurement with the different cats are shown in table 1, grouped according to the type of experiment. Under basal conditions the averages for the group varied in the eight periods from 0.76 to 0.77. The widest range in the average values for the individual periods was from 0.74 with A to 0.78 with O and W. There was only a slight tendency toward a fall in quotient in the 4 hours. The average respiratory quotients for 4 hours on the 6 different days of measurement on each cat varied from 0.73 to 0.78 with A, from 0.72 to 0.80 with M, from 0.72 to 0.82 with O, and from 0.73 to 0.81 with W. Variations in the basal values of the R. Q. of cats reported in the literature are somewhat the same as these or greater. The average values in table 1 agree most nearly with those of Ring ('35).

The effect on the R. Q. of the ingestion of water was only slight, on the average. This slight effect was most noticeable in general in the first  $\frac{1}{2}$ -hour period, although it persisted with G for the entire 4 hours and with W for 3 hours. The other cats showed little or no effect of the ingestion of water. In a few of the individual periods on particular days, the R. Q. values after water were lower than on the basal days.

The effect of the ingestion of 10 gm. of glucose was noticeable in all periods. The average R. Q. for this group of experiments was 0.78 in the first period, and the average peak was 0.89 in the sixth and seventh periods. Both A and G had higher R. Q. values, on the average and in individual experiments, than did the other three cats. These two animals were also the lowest in body weight. In two experiments with cat O and in one experiment with cat A the measurements were continued for  $7\frac{1}{2}$  hours. In these particular experiments the maximum R. Q. with A was 0.85 in the fourth, fifth, and sixth periods, but with O the

TABLE 1

Average respiratory quotients of cats as affected by ingestion of sugar.

CONDITION <sup>1</sup> AND CAT (1938-1939)	NO. OF EXPTS.	BODY WEIGHT (KG.)	HALF-HOUR PERIODS AFTER DOSE							
			1 <sup>2</sup>	2	3	4	5	6	7	8
Basal										
A	6	2.8	0.76	0.75	0.76	0.76	0.76	0.76	0.75	0.74
G	3	3.2	0.77	0.76	0.76	0.77	0.70	0.76	0.76	0.75
M	6	4.2	0.75	0.75	0.76	0.76	0.76	0.76	0.77	0.77
G	6	3.9	0.78	0.76	0.77	0.76	0.77	0.77	0.78	0.76
W	6	4.2	0.78	0.77	0.77	0.77	0.76	0.76	0.76	0.75
Water <sup>3</sup>										
A	5	3.1	0.77	0.76	0.77	0.76	0.75	0.75	0.75	0.74
G	2	3.5	0.78	...	0.80	0.79	0.78	0.78	0.78	0.78
M	6	4.3	0.76	0.76	0.77	0.77	0.77	0.77	0.77	0.75
O	6	4.0	0.77	0.75	0.75	0.75	0.75	0.74	0.75	0.73
W	5	4.3	0.82	0.79	0.79	0.78	0.77	0.77	0.76	0.75
Glucose <sup>4</sup>										
A	7	3.3	0.78	0.83	0.87	0.89	0.92	0.91	0.89	0.84
G	3	3.5	0.81	0.87	0.93	0.94	0.96	0.95	0.94	0.93
M	6	4.3	0.77	0.79	0.82	0.83	0.82	0.87	0.87	0.86
G	7	4.0	0.77	0.76	0.80	0.81	0.83	0.85	0.86	0.88
W	6	4.4	0.79	0.78	0.81	0.83	0.85	0.87	0.88	0.88
Fructose <sup>5</sup>										
A	6	3.3	0.76	0.81	0.85	0.86	0.86	0.84	0.82	0.79
G	4	3.5	0.78	0.85	0.87	0.88	0.87	0.87	0.86	0.85
M	6	4.4	0.74	0.77	0.82	0.83	0.83	0.83	0.85	0.82
G	6	4.0	0.77	0.76	0.78	0.79	0.80	0.81	0.82	0.81
W	7	4.5	0.80	0.77	0.79	0.81	0.82	0.81	0.81	0.80
Galactose <sup>4</sup>										
A	5	3.1	0.77	0.81	0.84	0.82	0.84	0.84	0.84	0.83
G	3	3.4	0.84	0.83	0.91	0.91	0.91	0.88	0.87	0.87
M	5	4.2	0.73	0.76	0.80	0.81	0.81	0.82	0.82	0.80
O	5	4.0	0.77	0.77	0.80	0.81	0.81	0.82	0.82	0.81
W	6	4.5	0.81	0.77	0.79	0.81	0.83	0.82	0.83	0.83
Sucrose <sup>4</sup>										
A	2	3.4	0.75	0.78	0.84	0.86	0.86	0.88	0.88	0.87
G	1	3.5	0.86	0.94	0.96	0.96	0.97	0.93	0.95	0.91
Maltose <sup>4</sup>										
A	2	3.5	0.78	0.80	0.85	0.87	0.91	0.90	0.90	0.86
M	3	4.1	0.75	0.75	0.78	0.80	0.80	0.82	0.85	0.84
Lactose <sup>4</sup>										
O	2	3.8	0.74	0.77	0.78	0.79	0.80	0.80	0.78	0.78
W	3	4.4	0.84	0.77	0.81	0.83	0.83	0.84	0.83	0.82
Glucose + galactose <sup>4</sup>										
W	3	4.5	0.80	0.77	0.81	0.84	0.86	0.85	0.88	0.88
Glucose + fructose <sup>4</sup>										
A	2	3.3	0.76	0.82	0.87	0.90	0.94	0.93	0.89	0.84

<sup>1</sup> Without food 16 to 24 hours at start of each experiment.<sup>2</sup> The first 1-hour period began about 20 mins. after the dose.<sup>3</sup> 75 ml. of water at 37°C.<sup>4</sup> 10 gm. of sugar in 50 ml. of water at 37° plus 25 ml. of water for rinse.<sup>5</sup> 5 gm. of each sugar in the usual amount of water.

maximum values were 0.92 and 0.89 in the tenth period. At the end of fifteen  $\frac{1}{2}$ -hour periods the R. Q. with A was 0.76 and with O was still 0.84. With cats, therefore, the effect of glucose can last at least  $7\frac{1}{2}$  hours.

The effect on the R. Q. of the ingestion of 10 gm. of fructose was noticeable by the second period and, in general, was at its peak (group average, 0.84) in the fifth period. Again the most marked effects were with A and G. In one experiment with O the measurements were continued for  $7\frac{1}{2}$  hours. At the end of that time the R. Q. was still 0.79. Hence 10 gm. of fructose may produce a rise in the R. Q. of the cat lasting nearly 8 hours. The rises in value of the R. Q. of these cats after fructose were not so large as those after glucose and were not nearly so large as those noted with man after 50 and 100 gm. of fructose. Also the peak effect with man usually occurs by the end of the first hour.

The rise in R. Q. after the ingestion of 10 gm. of galactose was noticeable in the second period and reached its peak, on the average, in the fifth, sixth, and seventh periods. Again A and G had the greatest rises, and with G the peak was 0.91, which is higher than the peak with this cat after fructose. One experiment with O of  $7\frac{1}{2}$  hours' duration gave an R. Q. of 0.80 in the last  $\frac{1}{2}$  hour. The rises in the R. Q. values of the cats were, on the whole, not so large nor so prompt as those with man after 50 gm. of galactose.

The ingestion of 10 gm. of sucrose was followed by a rise in R. Q. to 0.97 in the fifth period in one experiment with G and to an average of 0.88 in the sixth and seventh periods in two experiments with A. On the whole, when the experiments are assessed individually, it is evident that the rise was not so large as with glucose with either animal.

Maltose produced a greater rise in R. Q. in two experiments with A than did sucrose, although the rise was not so large as with glucose. In three experiments with M there was a rise in quotient with maltose but not so large as that with glucose.

The ingestion of 10 gm. of lactose was followed by a rise in R. Q. to an average maximum of 0.80 in the fifth and sixth periods in two experiments with O, and to 0.84 in the sixth period in three experiments with W. In the individual experiments with W there were maximum values of 0.88 and 0.87 in the sixth period. The general average for the three experiments with W is lowered by one experiment in which the average R. Q. for 4 hours was 0.78.

In three experiments with W with 5 gm. of glucose plus 5 gm. of galactose (approximately 10 gm. of lactose), the rise was as large as with glucose alone and larger than with galactose alone. Therefore, as has been found by others (Folin and Berglund, '22; Bodansky, '23; Harding and Grant, '33), the addition of glucose to galactose improved the metabolism of galactose.

The ingestion of an approximate equivalent to 10 gm. of sucrose (5 gm. each of glucose and fructose) in two experiments with A was followed by an average rise in R. Q. to 0.94 in the fifth period, which is even greater than the rise after ingestion of 10 gm. of glucose by the same animal. The effect of the combination of sugars was greater than that of 10 gm. of sucrose alone.

#### *Total metabolism and carbohydrate metabolized*

The average values for the total volumes of carbon dioxide eliminated and oxygen absorbed in the various groups of experiments with each cat are given in table 2. In general, these average values were lower under basal conditions than under the other conditions. No attempt has been made to assess the effects of ingestion of the sugars on the metabolism of energy. In most of the experiments the cats were quiet for the greater part of the time, but occasionally there were periods of marked activity. Study of the original protocols shows that it would be difficult to assess the values in relation to relative activity. Hence it seems best to report the total measurements as made, rather than to select periods arbitrarily and empirically and try to interpolate for the periods rejected. The values are therefore given as measured and can be used for purposes of calculation by other workers.

The main purpose of this study was to determine the effects of ingestion of various sugars upon the R. Q. and the metabolism of carbohydrates. Calculation of the amount of carbohydrate metabolized might be carried out simply by apportioning the metabolism between that of carbohydrate and that of fat, according to the total respiratory exchange. This method of calculation, however, would give too high values, because the metabolism of protein was not considered. Usually it is estimated that from 10 to 15% of the total energy output is derived from the metabolism of protein. As the cat is a carnivorous animal, it would under natural conditions have a predominantly protein metabolism. These animals were fed on canned salmon, mackerel, liver, beef, and milk. Therefore the proportion of the total metabolism represented by the protein metabolism must have been higher than

TABLE 2

*Total metabolism, R. Q., and calculated carbohydrate metabolism of cats as affected by ingestion of 10 gm. of sugar.*

*(Average values per 4 hours)*

CAT AND CONDITION	TOTAL METABOLISM		R. Q.		CARBO- HYDRATE METABO- LIZED
	CO <sub>2</sub>	O <sub>2</sub>	Total	Non- protein <sup>1</sup>	
	liters	liters			gm.
A					
Basal	3.92	5.20	0.75	0.71	0.0
Water	4.40	5.81	0.76	0.71	0.0
Glucose	4.75	5.49	0.87	0.92	2.4
Fructose	4.70	5.72	0.82	0.84	1.5
Galactose	4.72	5.72	0.83	0.84	1.6
Sucrose	4.14	4.93	0.84	0.88	1.5
Maltose	4.38	5.11	0.86	0.91	2.0
Glucose + fructose <sup>2</sup>	4.45	5.11	0.87	0.94	2.2
G					
Basal	4.95	6.51	0.76	0.71	0.0
Water	5.44	6.94	0.78	0.71	0.0
Glucose	7.02	7.68	0.91	1.13	4.9
Fructose	6.49	7.61	0.85	0.95	2.3
Galactose	6.62	7.49	0.88	1.05	3.7
Sucrose	7.09	7.60	0.93	1.20	5.2
M					
Basal	5.84	7.69	0.76	0.71	0.0
Water	5.69	7.46	0.76	0.71	0.0
Glucose	5.64	6.79	0.83	0.86	1.8
Fructose	5.99	7.39	0.81	0.81	1.4
Galactose	5.91	7.45	0.79	0.78	0.9
Maltose	4.85	6.07	0.80	0.78	0.6
O					
Basal	5.39	7.02	0.77	0.71	0.0
Water	5.29	7.05	0.75	0.71	0.0
Glucose	5.45	6.65	0.82	0.83	1.8
Fructose	5.80	7.31	0.79	0.78	1.3
Galactose	5.62	7.03	0.80	0.79	1.4
Lactose	4.75	6.09	0.78	0.75	0.6
W					
Basal	4.57	6.00	0.76	0.71	0.0
Water	5.34	6.85	0.78	0.71	0.0
Glucose	5.81	6.96	0.83	0.89	1.6
Fructose	6.10	7.61	0.80	0.79	0.9
Galactose	6.01	7.47	0.81	0.80	1.0
Lactose	5.59	6.81	0.82	0.85	1.2
Glucose + galactose <sup>2</sup>	5.51	6.59	0.84	0.91	1.5

<sup>1</sup> The average calculated protein combustion per 4 hours in basal and water expts., respectively, was: A, 2.37 and 2.84 gm.; G, 3.39 and 5.39 gm.; M, 4.05 and 4.11 gm.; O, 4.24 and 2.97 gm.; W, 3.27 and 4.97 gm.

<sup>2</sup> 5 gm. of each sugar.

15%. For purposes of calculation of the comparative amounts of carbohydrate metabolized after ingestion of the different sugars, the following assumptions have been made.

In the basal experiments and in the control experiments with water, as the R. Q. values were below 0.81, it has been assumed that the energy was derived from the metabolism of fat and protein alone. The average values for the protein combustion per 4 hours in the basal and the water experiments, as calculated on the basis of this assumption, are given in footnote 1 in table 2. It is recognized that they are wholly hypothetical and may not correspond to the facts. However, in view of the lack of information regarding the urinary nitrogen, the average value for each cat for the protein metabolized in the water experiments with this cat has been used as the basis of calculating the values of the non-protein R. Q. in the other groups of experiments with the same cat. The amounts of carbohydrate metabolized (table 2) have been calculated in the usual empirical manner from the non-protein gaseous exchange. In the three instances where the non-protein R. Q. values were greater than 1.00 (G after ingestion of glucose, galactose, and sucrose), the carbohydrate converted into fat has also been included in the values given in table 2, the calculation being made according to the method proposed by Lusk ('28).

Among the monosaccharides, glucose caused the greatest metabolism of carbohydrates in all five cats, galactose was next in order with four out of five cats, and fructose in general caused the least metabolism of carbohydrates. The disaccharides gave values that were sometimes higher and sometimes lower than the sum of the values for 5 gm. each of their constituent hexoses. The combination of glucose with one of the other hexoses to form a total amount of sugar equivalent to 10 gm. of sucrose or lactose gave results that were higher than would be expected by calculation of the sums of the effects of the sugars separately. This latter phenomenon was also observed with man after hexoses ingested separately and together.

In most of the experiments less than half of the 10 gm. of sugar was metabolized by any cat in the 4 hours of observation. Indeed, at the rate at which they were metabolized, many of the sugars would have supplied the metabolism of carbohydrates for over 24 hours. The serial order of the amounts of carbohydrate metabolized after the ingestion of these different sugars would be the same, regardless of the level of the metabolism of protein. Therefore the values given in table 2 serve for comparative purposes. It is apparent that cats have the ability to metabolize the disaccharides nearly as well as the mono-

saccharides, although two of the disaccharides, sucrose and maltose, are not common articles in the cat's diet in the natural state.'

#### SUMMARY

The respiratory exchange was measured in successive  $\frac{1}{2}$ -hour periods for 4 hours with five cats in the basal state, after ingestion of 75 ml. of water, after ingestion of 10 gm. of glucose, fructose, galactose, sucrose, maltose, or lactose, and after ingestion of a combination of 5 gm. each of glucose and fructose or of glucose and galactose.

The values of the basal R. Q. were uniform, for the most part, and did not show a marked tendency to change during the eight  $\frac{1}{2}$ -hour periods of measurement. The ingestion of water resulted in a rise in R. Q. with one cat for the entire 4 hours but with the other cats only in the first  $\frac{1}{2}$ -hour period. Glucose caused the greatest rise in R. Q., and the peak occurred in the sixth and seventh  $\frac{1}{2}$ -hour periods. All the other sugars, disaccharides as well as monosaccharides, caused definite rises in the R. Q.

On the assumption that in the control experiments with water only fat and protein were metabolized and that in the experiments with the sugars the protein metabolism of a given cat was the same as its average protein metabolism in the experiments with water, it was calculated that the metabolism of carbohydrates was highest after glucose and lower after galactose and fructose in the order named. The cats were able to metabolize the disaccharides nearly as well as would be expected, in view of their constituent monosaccharides formed by hydrolysis. When combinations of hexoses equivalent to 10 gm. of sucrose or lactose were ingested, the resultant metabolism of carbohydrates was greater than would be expected from the sum of the amounts metabolized after ingestion of the respective hexoses given separately.

Cats resemble men in the metabolism of the monosaccharides in that they show increases in R. Q. and in carbohydrate metabolism after ingestion of these sugars, but they differ from men in that the peak effect does not occur so promptly and, qualitatively, the order of magnitude of the effect is not the same.

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# THE AMINO ACID REQUIREMENTS OF THE CHICK

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## THREE FIGURES

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Previous studies of the amino acid requirements of the chick have been carried out by using diets containing ingredients which are poor sources of a certain amino acid, or by removing one or more of the amino acids after acid hydrolysis. By using these methods, it has been shown that the chick must have dietary sources of arginine, glycine, histidine, tryptophane, lysine and methionine (see review by Almquist, '42, also Grau and Almquist, '43) and isoleucine (Grau and Almquist, '44). In a continuation of these studies, mixtures of pure amino acids have been used in complete replacement of all proteins in the diet. Some of the amino acids which have already been shown to be required by the chick (glycine, histidine and isoleucine) have been reinvestigated with diets containing the amino acid mixtures; the other recognized amino acids which have been studied include alanine, aspartic acid, glutamic acid, hydroxyproline, leucine, norleucine, phenylalanine, proline, serine, threonine, tyrosine and valine. The tryptophane requirement will be discussed elsewhere.

## EXPERIMENTAL

White Leghorn chicks, which had been reared on a commercial-type chick mash for 10-14 days, were maintained as reported previously (Grau and Almquist, '43). The basal ration was as follows: cellulose<sup>1</sup> 5, calcium gluconate 8, mineral mixture 3.24, crude soybean oil 5, sardine oil (400D-3000A per gram) to which 1% of mixed tocopherols<sup>2</sup> had been added 0.25, choline chloride 0.2, inositol 0.1, cholic acid 0.1, 2-methyl-1, 4-naphthohydroquinone diacetate 0.001, thiamine 0.001, riboflavin 0.001, pyridoxine 0.001, nicotinic acid 0.003, calcium pantothenate (*dL*) 0.003, biotin (acid)<sup>3</sup> 0.00001 gm., with the amino acid mixture and glucose<sup>4</sup>

<sup>1</sup> Cellu Flour.

<sup>2</sup> Natural Mixed Tocopherols, Type I (Distillation Products, Inc.).

<sup>3</sup> The biotin was kindly provided by Merck and Co., Inc. through the courtesy of Dr. J. C. Keresteszy.

<sup>4</sup> Cerelease.

to make 100 gm. Other members of the vitamin B-complex were provided by a concentrate prepared from solubilized liver which added only 4 mg. nitrogen to each 100 gm. diet. The mineral mixture furnished the following: tricalcium phosphate 2000, dipotassium phosphate 500, potassium chloride 300, and manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8 and cobalt 0.5 mg. per 100 gm. diet.

TABLE 1

*Mixture of amino acids used in the positive control diet.*

AMINO ACID	FORM OF ACID	LEVEL AS FED	LEVEL OF NATURAL ISOMER	AMINO ACID	FORM OF ACID	LEVEL AS FED	LEVEL OF NATURAL ISOMER
		%	%			%	%
Alanine	dl	1.0	0.5	Methionine	dl	1.0	0.5
Arginine	1(+)-HCl	1.4	1.2	Norleucine	dl	0.2	0.1
Aspartic acid	1(+)	2.0	2.0	Phenylalanine	dl	1.0	0.5
Cystine	1(-)	0.4	0.4	Proline	1(-)	2.0	2.0
Glutamic acid	1(+)	5.0	5.0	Serine	dl	0.4	0.2
Glycine	....	1.8	1.8	Threonine	dl	3.0	1.5
Histidine	1(+)-HCl·H <sub>2</sub> O	0.8	0.6	Tryptophane	1(-)	0.4	0.4
Hydroxyproline	1(-)	0.2	0.2	Tyrosine	1(-)	2.0	2.0
Isoleucine	dl	2.0	1.0	Valine	dl	2.0	1.0
Leucine	1(-)	2.0	2.0				
Lysine	1(+)-HCl	1.4	1.1	Total		30.0	24.0
				(Sodium bicarbonate)		1.5	

The amino acids used in these studies were commercial products. The levels used in the diets and the form in which they were added are summarized in table 1. Glucose replaced the omitted amino acids except for the glutamic acid studies, where aspartic acid was added. The sodium bicarbonate was added to neutralize the hydrochlorides of the basic amino acids and to provide sodium chloride, which was omitted from the mineral mixture generally used.

## RESULTS

*Alanine.* Four chicks maintained for 10 days on a diet lacking alanine gained in weight just as well as a similar group which was fed alanine. Alanine is apparently completely dispensable for chick growth.

*Aspartic acid.* In the first experiment dealing with this amino acid, glutamic acid as well as aspartic acid was omitted, and no attempt was made to replace this loss of amino acid nitrogen. This group of four chicks lost weight for 2 days, then began to gain at an increasing rate, so that from the time of minimum weight until 10 days later, the gain

per day<sup>5</sup> was 2.4%. Over a similar period, the positive control group gained at a rate of 4.0%. In a later experiment, where aspartic acid alone was omitted, the growth rate obtained with three chicks for 14 days was the same as that of the positive control. These data indicate that aspartic acid is a dispensable component of the diet of the chick.

*Glutamic acid.* Besides the experiment mentioned above, in which both of the dicarboxylic acids were omitted, three chicks were kept for 14 days on a diet containing extra aspartic acid, but no glutamic acid.

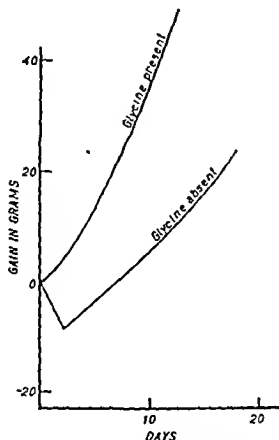


Fig. 1 Effect of omission of glycine from the amino acid mixture on the growth of chicks.

The total l (+)- aspartic acid content of the diet was 4.0% instead of the usual 2.0%. These chicks reacted in the same way as those which received neither aspartic acid nor glutamic acid, showing an original weight loss, and a slow gain thereafter. Because of the indecisive nature of these results, it can only be said that lack of glutamic acid causes slower gains than those obtained with control groups, and that if glutamic acid is required at all in the diet, it is needed only to promote most efficient growth.

*Glycine (fig. 1).* That the chick is able to carry on definite, but limited glycine synthesis was suggested when casein diets containing low levels of glycine gave a moderate growth which was increased markedly by

<sup>5</sup> Average per cent gain per day =  $\frac{\text{Average gain} \times 100}{\text{No. days on expt} \times \text{avg wt. during expt.}}$

additions of glycine to the diet (Almquist and Mecchi, '42). Results with four chicks on a glycine-deficient amino acid mixture for 18 days showed poor growth and gain per gram of food eaten. Omission or inclusion of serine had no effect on the glycine deficiency. These results with diets free from glycine confirm the previous conclusion that the chick can synthesize glycine, but not at a rate high enough to allow best growth.

*Histidine.* Four chicks which received a histidine-deficient diet lost weight for the first 4 days of the experiment and remained at this low weight level for the remainder of the 10-day period. Other chicks which received 0.15% *l* (+)-histidine (fed as the monohydrochloride monohydrate) showed gains which indicated that most of the requirement for this amino acid had been met. Histidine is, therefore, an indispensable amino acid.

*Isoleucine.* A group of three chicks fed a diet deficient in isoleucine lost weight and died, confirming the previous investigations in which dried beef blood cell protein was used as the sole protein source (Grau and Almquist, '44), and in which the necessity of isoleucine in the diet was proved.

*Leucine.* A preliminary experiment with three chicks indicated that chicks lost weight rapidly on leucine-deficient diets, and that while either 4.0% of the *dl* form or 2.0% of the *l* (—) form was adequate, 1.0% of the *l* (—) form was inadequate for best growth. Another experiment with three chicks maintained for 16 days showed a rapid weight loss followed by a more gradual loss; the curve was practically identical with that obtained with phenylalanine or threonine deficiencies (figs. 2 and 3). Leucine is an indispensable component of the diet.

*Norleucine.* Omission of norleucine from the diet appeared to have no adverse effect on the rate of gain of three chicks which were maintained for 16 days. This amino acid is apparently completely dispensable for the chick.

*Phenylalanine.* Four chicks on a phenylalanine-deficient diet lost weight rapidly (fig. 2) when the diet contained 2.0% *l* (—)-tyrosine, but gained at the normal rate when 1.0% *dl*-phenylalanine was present in addition. Indicated interrelationships between these two amino acids are discussed under tyrosine.

*Proline and hydroxyproline.* These amino acids were omitted from the diet of four chicks to determine if either one was necessary. The chicks maintained their weight for the first 4 days, but did not grow; after that time, they grew at the normal rate. This apparent time lag may have been required to allow establishment of adequate synthesis of

these amino acids from others furnished in the diet. In any case, the omission of the prolines had no lasting effect on the growth rate; hence, they may be classified as dispensable amino acids.

*Serine.* Unlike glycine, serine is a completely dispensable component of the diet of the chick, as was shown in the normal growth of four chicks maintained for 8 days on a serine-deficient ration. As mentioned above, serine has no ability to replace glycine as an essential dietary component.

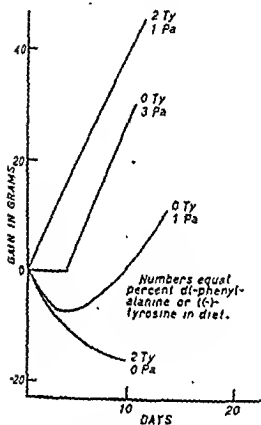


Figure 2

Fig. 2 Curves showing the effects of phenylalanine and tyrosine deficiencies on the growth of chicks.

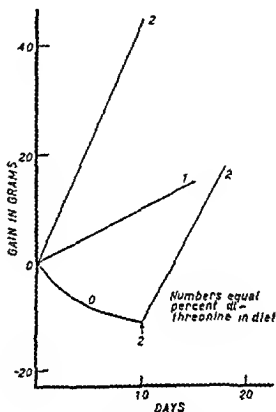


Figure 3

Fig. 3 Effect of threonine deficiency on chick growth.

*Threonine.* Some of the results with threonine are shown in figure 3, where the recovery from an amino acid deficiency is shown to occur immediately after supplying the missing substance. The change from weight loss to gain in 24-48 hours is often a clear indication of the future course of such an experiment. This early weight gain is primarily a reflection of the increased appetite which is caused by the change in diet: fresh diet alone will not evince the effect. Feeding the *dl* form at 1.0% of the diet evidently is not enough for best growth, and, while 2.0% appears adequate, 3.0% was fed for safety until the most satisfactory level could be determined. In another experiment with two chicks, a threonine-deficient diet was fed for 27 days, at which time

one of the chicks died, with no apparent symptoms other than those which would occur with malnutrition or starvation.

*Tyrosine.* The absolute status of tyrosine cannot be determined from the data to be presented here, but must await further study. As shown in figure 2, addition of tyrosine is required to allow best growth if only 1.0% of the *dl*-phenylalanine is present in the diet, but 3.0% *dl*-phenylalanine alone appears to obviate the need for additional tyrosine. Tentatively, then, it may be said that tyrosine is required to promote optimal growth if phenylalanine is present at only a moderate level in the diet.

*Valine.* Of all the deficiencies studied, valine and isoleucine seem to exert the most profound effects on chicks; however, none of the peculiarities of gait or sensitiveness to touch, such as were observed with rats (Rose, '38), have been noted. The rapid and continuing decline in weight with valine deficiency was almost identical with that observed with phenylalanine deficiency (fig. 2). Three of the four chicks on one valine-deficient diet died on the twelfth day of the experiment, and all were extremely weak for a few days before this time. Addition of either 2.0% isovaline (*dl*- $\alpha$ -amino- $\alpha$ -methylbutyric acid) or 2.0% norvaline (*dl*- $\alpha$ -amino-n-valeric acid) did not diminish the severity of the deficiency. After 6 days, *dl*-valine was added. Within 24 hours, all chicks had gained in weight, and they continued to do so until the experiment was terminated 8 days later.

#### SUMMARY

Mixtures of 20 amino acids have been used in complete replacement of protein in chick diets. Of these amino acids, alanine, aspartic acid, hydroxyproline, proline, norleucine and serine have been shown to be dispensable. The essential nature of glycine in promoting best growth has been confirmed; glutamic acid appears similar to glycine in this regard. Tyrosine appears to be required if only moderate levels of phenylalanine are used, but seems to be dispensable when higher levels are used. Leucine, phenylalanine, threonine and valine have been shown to be necessary dietary components to prevent weight loss, while the similar status of histidine and isoleucine has been confirmed.

#### ACKNOWLEDGMENT

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# DENTAL CARIES IN THE COTTON RAT<sup>1</sup>

## I. METHODS OF STUDY AND PRELIMINARY NUTRITIONAL EXPERIMENTS

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### ONE FIGURE

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The vitamin B complex requirements of the cotton rat (*Sigmondon hispidus hispidus*) have been described by McIntire, Schweigert and Elvehjem ('44). In the course of the experiments in which these requirements were evaluated, a preliminary survey was made to determine how susceptible the teeth of the cotton rat were to decay. It was found that the molar teeth of the cotton rats which were raised from weaning on synthetic, high sucrose rations were highly susceptible to decay. On the contrary, the molar teeth of cotton rats which were on stock rations composed of crude materials were highly resistant to decay. Therefore, the various experiments reported here were devised to study the factors involved in the incidence and progress of the carious lesions in the molars of the cotton rat.

### EXPERIMENTAL

The cotton rats used in these experiments were obtained from our stock colony which was maintained on the Steenbock stock ration no. 14<sup>2</sup> supplemented with greens. These animals were weaned at the age of 3 weeks and at a weight of 20 to 25 gm.

The basal ration (801) consisted of: Labco casein 18%, sucrose 73%, salts IV 4% (Phillips and Hart, '35) and corn oil 5%. To each 100 gm. of ration was added: thiamine 250 µg., riboflavin 300 µg., nicotinic acid

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We are indebted to Merck and Co., Rahway, New Jersey for the synthetic vitamins; to Abbott Laboratories, North Chicago, Illinois for halibut liver oil; and to Wilson and Company, Chicago, Illinois, for the liver preparations.

<sup>2</sup>Ground corn 71.5%, linseed oil meal 15.0%, alfalfa leaf meal 2.0%, CaHPO<sub>4</sub> 1.0%, NaCl 0.5%, crude casein 5.0% and butter fat 5.0%.

2.5 mg., pyridoxine 250  $\mu$ g., pantothenic acid 2 mg., choline 100 mg., inositol 100 mg., and p-aminobenzoic acid 30 mg.; 1 drop of haliver oil was fed weekly. Later a modification (802) of the basal ration was found to give more uniform growth. It consisted of an increase in the casein to 24% at the expense of the sucrose. .

In early experiments the cotton rats were left on experiment for periods ranging from 10 to 14 weeks to determine the optimum experimental period. For accurate results it was found necessary to sacrifice the animals at a time when the carious lesions were large enough to be readily detected and yet not so large that any extensive amount of fracturing of the cusps had occurred. It is especially necessary to have a minimum amount of fracturing. Old widespread fractured areas may overlie and involve several of the original carious lesions. Thus any accurate evaluation of the numbers of lesions and the extent of each lesion would be impossible. In preliminary studies it was found that the most suitable experimental period lay between 10 to 14 weeks after weaning. When 14 weeks was established as the optimum period, all animals were sacrificed at that age.

Various liver preparations (1:20 liver extract, alcohol ether extract of 1:20 liver extract, solubilized liver, and whole liver substance) were added to the basal ration to determine whether the more rapid rate of growth thus obtained would have any effect upon the incidence and progress of carious lesions.

In order to determine whether or not additional fat-soluble vitamins would have any effect on the development of the carious lesions, these vitamins were fed to one group at the following levels per 100 gm. of ration: 500  $\mu$ g. of  $\beta$ -carotene, 12.5  $\mu$ g. of calciferol, 2 mg. of  $\alpha$ -tocopherol, and 187.5  $\mu$ g. of 2 methyl, 1, 4 naphthoquinone. These vitamins were kept in chloroform in the refrigerator. When the ration was to be mixed, the necessary amount of the chloroform solution was evaporated to dryness and the vitamins taken up in corn oil so that 5 gm. of corn oil contained the amount of these vitamins to be added to 100 gm. of ration. This corn oil was then incorporated into the ration in the customary fashion.

In order to compare the effect of particle size of the diet upon the incidence of carious lesions two procedures were followed. The first comparison was made with Steenbock stock ration no. 14 in which the particles of corn used in its composition would pass through a 20 mesh, but not through a 40 mesh sieve. A portion of this ration was then ground in a ball mill until all would pass through a 60 mesh sieve. The coarse ration was fed to one group, while the fine was fed to a second

group. The second comparison was made by the substitution of dextrin for sucrose in ration 801. The particle size of dextrin used in the coarse ration was such that it would pass through a 20, but not through a 40 mesh sieve; and that in the fine ration would all pass through a 60 mesh sieve.

At the end of the experimental period, the animals were sacrificed. The heads were fixed in 95% ethanol for 24 to 48 hours. They were then skinned and the flesh removed. The jaws were placed in fresh 95% ethanol and stored until observations could be made. The observations on the molar teeth reported here were made upon successive planes ground parallel to the occlusal surface by means of a vertically rotating carborundum wheel. After each grinding, the newly exposed plane was observed at a magnification of 30 diameters with a binocular dissecting microscope. Each lesion observed was carefully explored with a no. 42 fine, smooth dental broach to determine the depth of softening, as a supplement to the visual changes in color and opalescence.

## RESULTS

### *Description of the dentition of the cotton rat*

The dentition of the cotton rat is similar to that of the white rat: monophyodont, one incisor and three molars in each quadrant of the jaws, continuously erupting incisors, and molars which are more highly developed than the incisors and which are limited in development to the early period of life. The molars are similar to those of the white rat in most respects, but have a few notable differences. When the molars of the cotton rat are erupted, there are definite cusps as in the white rat. But in the cotton rat the completely transverse occlusal fissures between the cusps are very shallow. Attrition of the cusps exposes small circular areas of dentin which become larger as the cusp is more deeply abraded. Since enamel is more resistant to attrition, the enamel rim is perceptibly higher than the exposed dentin.

After a period of 3 or 4 weeks of occlusal contact, sufficient attrition has occurred that the cusps have been abraded to a level below the bottom of the shallow transverse occlusal fissures. Then all the cusps of any molar become connected at the occlusal surface. The exposed dentin area of a cusp is connected to the similar area in the next anterior or posterior cusp by a narrow strip of exposed dentin, flanked on each side by enamel. Thus in the cotton rat, a few weeks after the eruption of any molar, its occlusal surface has a continuous region of exposed dentin extending from the anterior cusp to the posterior cusp. This can be

seen in figure 1 (except in the third molars of the upper jaw which are not fully erupted).

The occlusal fissures which are present in the fully erupted molar extend from  $\frac{1}{3}$  to  $\frac{2}{3}$  across the tooth from either the labial or lingual surface. These fissures are exceedingly narrow and extend from the occlusal surface of the molar almost to the cemento-enamel junction.

### *Regions of tooth decay*

In the cotton rat it was found that tooth decay occurred almost exclusively in the occlusal fissures and at such a depth that early decay could not be observed in the intact tooth because of the depth and narrowness of these fissures. When decay had progressed sufficiently, regions of the cusps were broken away due to the undermining effect of these deeply set carious regions. Only then, was it possible to determine from the occlusal surface that decay had been occurring in the occlusal fissures. Hence, in order to study and evaluate decay in the teeth of the cotton rat, it was necessary to use an alternate grinding and observation procedure similar to that suggested by Cox and Dixon ('39).

A very low incidence of decay was found on the proximal surfaces of the molars or on the exposed occlusal surfaces. The rarest location where decay was found was along the gingival margin. However, where decay was found in these regions the development and the progress of the lesions was entirely comparable to the lesions found in the narrow occlusal fissures.

The first indication of decay was a slight darkening of the enamel, and a decrease in its opalescence. This was accompanied by a softening of the affected area. This early lesion became quite evident when it was still less than 0.1 mm. in diameter. At that time the enamel was softened sufficiently that a no. 42 fine, smooth dental broach would readily penetrate a short distance into the lesion. The destruction of the enamel apparently was quite rapid once the lesion had been initiated. Frequently an area 0.2 to 0.3 mm. in diameter would have become decayed before any appreciable amount of penetration into the dentin had occurred. Once the dentin had become affected the lesion spread more rapidly until large areas of the cusp were involved. About this time fracture of the undermined crown of the cusp began. Fracture of large portions of the cusp did not usually occur immediately but instead small portions broke off as they were undermined. There is ample evidence for this statement since many lesions occurred where there was a loss of only small portions of a cusp due to fracture. Fracture continued progressively as decay progressed until finally the entire

portion of the cusp affected was undermined and broken off; after fracture occurred, decay continued in the original lesion at about the same rate. The surface exposed by the fracture did not appear to be attacked by the process of decay at any greater rate than if fracture had not occurred. Instead decay progressed into this exposed face from the original lesion as if no fracture had occurred.

*The method of recording position and evaluating the extent of the carious lesions*

In order to record the number of decayed areas in such a way as to be able to study the frequency in any given area, the various occlusal fissures were numbered in a manner similar to that developed by Cox, Dodds, Dixon and Matuschak ('39) for use in the evaluation of corn meal caries in the white rat. It was not possible to use the identical numbering system of Cox et al. since the cotton rat differs from the white rat in too many anatomical details. Figure 1 shows the numbering system used for the cotton rat.

An arbitrary system for the evaluation of the extent to which the decay had progressed in any lesion was devised. The system employed symbols 1 + to 5 + to represent the increasing extent of the lesions, as follows: 1 + — a lesion where there was a small, dark, definitely softened decayed region in the enamel without any penetration of the dentin; 2 + — a lesion with a more widespread softening of the enamel and a slight penetration into the dentin; 3 + — a lesion with widespread decay of the enamel and deep penetration of the dentin but without fracture; 4 + — a lesion with widespread decay in both the enamel and the dentin and a small amount of fracture of the undermined cusp; 5 + — a lesion which has progressed so far that a large amount, or all, of the undermined portion was broken off.

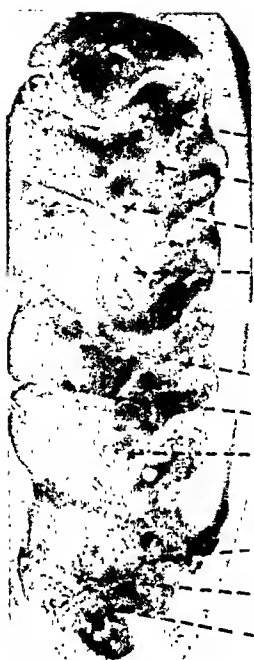
For each cotton rat studied the evaluations assigned for the individual carious lesions were totaled. This sum was used as an index of the extent to which decay had progressed in each animal. The average amount of decay for each carious lesion was obtained by dividing this sum by the number of lesions observed.

This type of evaluation of the extent of the lesions was at best an arbitrary one, but a very essential one. There could be no absolute unit of measurement for the progress of decay. Any evaluation assigned by the observer was, of necessity, a measurement of degree which took into account the numerous changes observed in color, texture, and size of the lesions. Therefore, by the system outlined, we have a satisfac-

# UPPER

Left

Right



- 1
- 3
- 5
- 7
- 9
- 11
- 13
- 15
- 17
- 19

I

II

III

- 2
- 4
- 6
- 8
- 10
- 12
- 14
- 16
- 18
- 20



# LOWER



- 21
- 23
- 25
- 27
- 29
- 31
- 33
- 35
- 37
- 39

I

II

III

- 22
- 24
- 26
- 28
- 30
- 32
- 34
- 36
- 38
- 40

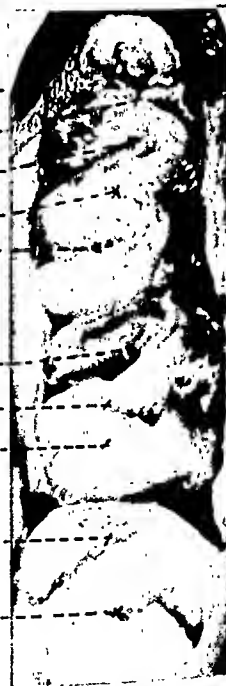


Fig. 1 Occlusal surfaces of the molars of a cotton rat labelled to indicate the occlusal fissures in which the carious lesions occur. This rat was one which had been raised on the stock ration and was entirely free of tooth decay.  $\times 16$ .

tory and consistent method of evaluating the degree of the carious processes in any animal or group of animals.

*The effect of the length of experimental period upon the development of the carious lesions*

The observations from the cotton rats which were sacrificed at 10, 12, and 14 weeks to determine the most suitable length of experimental period are presented in table 1. As the duration of the experimental period was increased, there was observed in the cotton rats on each ration, a definite increase in the number of carious lesions per animal, a definite increase in the total extent of the carious lesions and a definite

TABLE 1

*The effect of the length of experimental period upon the development of carious lesions in the cotton rat.*

RATION	NO. OF WEEKS	NO. OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS	AVERAGE EXTENT OF CARIOUS LESIONS	AVERAGE NUMBER OF CARIOUS LESIONS		AVERAGE EXTENT OF CARIOUS LESIONS	
					Upper jaw	Lower jaw	Upper jaw	Lower jaw
801	10	3	11.8	25.8 +	2.8	9.0	5.8 +	20.0 +
	12	3	21.3	53.0 +	8.6	12.7	16.7 +	36.3 +
	14	2	24.5	78.0 +	10.0	14.5	27.5 +	50.5 +
801 + 4% 1:20 L.E.	10	4	12.0	25.3 +	3.0	9.0	5.5 +	19.8 +
	14	7	26.0	74.3 +	12.1	13.0	32.0 +	42.3 +
802	12	5	22.8	42.2 +	9.8	13.0	19.0 +	23.2 +
	14	4	27.3	63.5 +	12.5	16.0	22.5 +	41.0 +
Coarse stock	12	3	6.0	12.0 +	0.7	5.3	0.7 +	11.3 +
	14	4	6.8	15.5 +	2.0	4.8	2.0 +	13.8 +

increase in the average extent of decay in each carious lesion. It appeared that under our present experimental regime the optimum experimental period was 14 weeks. At that age the carious lesions in the molars had progressed to the point where they were readily visible when exposed by grinding. Yet only a small amount of fracture had occurred. If the experimental period were longer the fracturing would have become so extensive that many individual lesions would have been obscured.

*The distribution of the carious lesions in the various molars*

In these early experiments there was noted a very conspicuous difference between the incidence of carious lesions in the upper and lower



jaws. In figure 1 it is seen that in both the upper and lower jaws there are 10 occlusal fissures in the three molars of each quadrant. Since, as we have reported above, it was in the occlusal fissures that almost all decay was initiated in the cotton rat molars, each quadrant had an equal number of susceptible regions. However, it was found throughout all the groups of animals studied that there was a much lower incidence of carious lesions and a smaller amount of decay in each lesion in the molars of the upper jaw than in those of the lower jaw (table 1). Once initiated the rate of development of the carious lesions in the molars of the upper jaw appeared to be comparable to that in the lower jaw.

TABLE 2

*A summary of the relative incidence and extent of carious lesions in the molars of 65 cotton rats which had been maintained on sucrose rations for 14 weeks.*

JAW	MOLAR	TOTAL NUMBER OF CARIOUS LESIONS (A)	TOTAL EXTENT OF CARIOUS LESIONS (B)	AVERAGE EXTENT OF EACH CARIOUS LESION $\left(\frac{B}{A}\right)$	NUMBER OF CARIOUS LESIONS PER MOLAR $\left(\frac{A}{2 \times 65}\right)$	NUMBER OF SUS- CEPTIBLE REGIONS PER MOLAR (C)	PER CENT OF SUSCEPTIBLE REGIONS THAT DEVELOPED CARIOUS LESIONS $\left(\frac{A}{2 \times 65} \times \frac{1}{C} \times 100\right)$
Upper	First	122	194 +	1.6 +	.9	4	23
	Second	299	797 +	2.7 +	2.3	3	77
	Third	200	436 +	2.2 +	1.5	3	51
Lower	First	305	632 +	2.1 +	2.3	5	47
	Second	326	952 +	2.9 +	2.5	3	84
	Third	239	737 +	3.1 +	1.8	2	92

Since the lesions in the upper jaw were initiated slightly later than those in the lower jaw, the extent of decay in each of these lesions was always slightly less than in those in the lower jaw at any given time of observation.

Very definite differences were seen in the rate of incidence of carious lesions in the three molars of both the upper and lower quadrants. A summary was made of the number of carious lesions observed in each molar of 65 cotton rats which had been maintained on synthetic rations for 14 weeks. The results of this summary are presented in table 2. It was found that there was an increasing rate of incidence and also an increasing extent of decay in each lesion of the molars in the following order: upper first, lower first, upper third, upper second, lower second, and lower third. The upper molar in each case had a lower rate of incidence of carious lesions and each lesion had a lesser extent of decay than the respective lower molar.

Since the first molars were fully erupted and the second molars almost fully erupted at the beginning of the experiment, they were subjected to the conditions of the oral cavity for about the same length of time. Therefore, if all the factors influencing decay were the same, the first and second molars of all four quadrants would be equally affected. However, from the summary it can easily be seen that there was a very much higher rate of incidence of carious lesions in the second molars than in the first molars of the upper and lower jaws. This different rate of incidence may be affected by one or more of several factors: the structure and topography of the molars themselves and the position of the molars in the mouth.

The lower third molar was erupted after the animals had been on experiment for about 7 weeks. The upper third molar was erupted after about 10 weeks of the experimental period had elapsed. The time of eruption of the upper and lower third molars varied with the rate of growth. The more nearly complete the ration was nutritionally, the more rapidly the animals grew and the sooner the upper and lower third molars were erupted. However, the lower third molars were only exposed to the environment of the oral cavity for about one-half and the upper third molars for only about one-third of the experimental period. The very high incidence of carious lesions in the lower third molars had added significance when this short period of exposure was considered. Although the rate of incidence of carious lesions in the upper third molars was lower than in the lower third molars, the very short period of exposure indicated a high susceptibility to decay. It appeared that the third molars were much more susceptible to decay in the cotton rat than either the first or the second molars. This may be due to the relative position in the mouth, or the structure of the third molars may have been unfavorably affected during the development by some deficiency or deficiencies in the experimental rations.

*The effect of diet upon the rate of incidence and the extent of the carious lesions*

There was a high rate of incidence of carious lesions in the molars of the cotton rats fed the two basal synthetic rations 801 and 802 (table 3). The additional 6% casein contained in the latter ration did not decrease the rate of incidence nor the extent of the carious lesions in any way. Our groups of animals were small in all cases reported since only a limited number of animals was available because of our small stock colony. However, in every case the caries incidence and extent did not vary to any appreciable extent within any one group or between groups

when an experimental series was repeated. Therefore, the differences reported here were of importance.

The effect upon the incidence and extent of the carious lesions of various supplements to the synthetic rations is presented in table 3. It was observed that the addition of 4% 1:20 liver extract, alcohol ether extract equivalent to 6 or 9% of 1:20 liver extract, 4% solubilized liver or 4% whole liver substance to synthetic ration 801 did not alter the rate of incidence or the extent of carious lesions. When additional

TABLE 3

*The effect of various supplements to synthetic rations upon the incidence of carious lesions in the cotton rat.*

RATION	NUMBER OF WEEKS	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS (D)	AVERAGE EXTENT OF CARIOUS LESIONS (E)	AVERAGE EXTENT OF EACH CARIOUS LESION ( $\frac{E}{D}$ )
801	14	2	26.0	70.0 +	2.7 +
801 + 4% 1:20 L. E.	14	7	26.0	74.3 +	2.9 +
801 + alcohol ether extract equiv. to 6% 1:20 L. E.	14	2	25.5	64.5 +	2.6 +
801 + alcohol ether extract equiv. to 9% 1:20 L. E.	14	4	23.8	72.3 +	3.0 +
801 + 4% solubilized liver	14	2	29.5	73.5 +	2.5 +
801 + additional vitamins A, D, E, and K	14	4	20.3	36.5 +	1.3 +
801 + 4% whole liver substance	12	2	24.5	63.0 +	2.6 +
802	14	4	27.3	63.5 +	2.3 +
802 + 4% 1:20 L. E.	14	3	27.7	62.0 +	2.2 +
802 + alcohol extract equiv. to 6% 1:20 L. E.	14	3	27.0	63.3 +	2.3 +

amounts of the fat-soluble vitamins, A, D, E, and K were fed as supplements to ration 801, there was a slight reduction in the rate of incidence and a very substantial decrease in the rate of progress of decay in the lesions. Studies are now in progress to determine which of the fat-soluble vitamins was responsible for this decrease in rate and extent. This result may be similar to the effect attributed to vitamin K by Fosdick, Fancher and Calandra ('42). However, Armstrong, and Knutson ('43) and Armstrong, Spink and Kahnke ('43) explained the effect reported by Fosdick et al. as an effect due to quinones in general rather than any specific inhibiting action of vitamin K.

A supplement of 4% 1:20 liver extract or of an alcohol extract equivalent to 6% of 1:20 liver extract to ration 802 did not have any effect upon the rate of incidence or the extent of the carious lesions.

Both the Steenbock stock ration no. 14 and the ration in which dextrin was substituted for sucrose produced an almost complete prevention of carious lesions in the cotton rat molars (table 4). This was in contrast to the high incidence of carious lesions observed on the sucrose rations.

TABLE 4

*The effect of coarse and fine rations upon the incidence of carious lesions in the cotton rat.*

RATION	NUMBER OF WEEKS	NUMBER OF ANIMALS	AVERAGE NUMBER OF CARIOUS LESIONS (D)	AVERAGE EXTENT OF CARIOUS LESIONS (E)	AVERAGE EXTENT OF EACH CARIOUS LESION $\left(\frac{E}{D}\right)$
Coarse stock	12	3	6	12.0 +	2.0 +
Fine stock	12	3	6	8.0 +	1.3 +
Coarse dextrin	12	3	2	2.0 +	1.0 +
Fine dextrin	12	1	1	1.0 +	1.0 +
Coarse stock	50	23	3	7.6 +	2.5 +

*The effect of particle size of the diet upon the incidence of carious lesions*

The results obtained as a comparison of the effect of particle size on the incidence of carious lesions is presented in table 4. Difficulties were immediately encountered in the groups of animals fed either of the finely ground rations. The cotton rats almost completely refused to eat either of the fine rations. Instead they scattered it out of the food cup immediately after the daily feeding. Therefore, few of these animals survived the 14-week experimental period. In an effort to obtain as much data as possible, the surviving members were sacrificed at 12 weeks. It was found after that experimental period that no differences resulted in the rate of incidence of carious lesions when the particle size was varied. There was equal protection from decay in the molars on either the coarse or fine stock and dextrin rations.

#### DISCUSSION

The cotton rat is unquestionably more susceptible to tooth decay than any other experimental animal yet reported. The very high incidence of carious lesions in the molar teeth of the cotton rat raised on a high sucrose ration was in great contrast to the extremely low incidence observed when the sucrose of the ration was replaced by coarse or fine

dextrin. A similarly low incidence was observed in those animals fed either a coarse or fine stock ration. Since particle size did not appear to play any role in the development of the carious lesions in the molars of the cotton rat, this uncontrollable variable in the study of dental caries in the white rat was not encountered. Since the cotton rat is very susceptible to dental caries when fed certain rations, but extremely immune when on others, it can be used for many types of dental caries research. When such a wide difference in caries incidence is possible, the positive or negative value of the treatment given during the experimental period can be readily and accurately evaluated.

As yet only the supplement of additional vitamins A, D, E, and K, was found to have any preventative effect when a high level of sucrose was fed in the ration. However, this protective effect was much less than that observed when sucrose was replaced by dextrin or when a stock ration of common foodstuffs was fed. Studies are being conducted to determine which of these vitamins was responsible and if the effect was due to that particular vitamin or to the class of compounds to which it belongs.

There was an almost complete bilaterally equal distribution of the carious lesions in both the upper and lower jaws. This has been shown to exist in man by Bertram and Brown ('43).

The upper jaw has a very much lower incidence of carious lesions than the lower jaw. This has been reported by Klein and Palmer ('41) for the first and second permanent molars of the human.

#### SUMMARY

1. The cotton rat is highly susceptible to tooth decay when fed a synthetic ration high in sucrose.

2. Almost no tooth decay occurred in those cotton rats which were fed a stock ration composed of natural foodstuffs or a synthetic ration in which the sucrose had been replaced by dextrin.

3. An experimental period of 14 weeks was found to be optimal for the observation and evaluation of the carious lesions.

4. No differences in the rate of incidence or the extent of the carious lesions could be produced by altering the particle size of the stock or dextrin rations.

5. The various liver supplements which were added to the sucrose rations to produce optimal growth had no effect on the development of the carious lesions.

6. Increased amounts of the fat-soluble vitamins, A, D, E, and K, produced a decrease in the number and the extent of the carious lesions in the molars of cotton rats on the sucrose ration 801.

7. There was a definite bilateral distribution of the carious lesions. There was a much lower rate of incidence of carious lesions in the molars of the upper jaw than in those of the lower jaw. The ascending order of caries incidence in the molars was as follows: upper first, lower first, upper third, upper second, lower second and lower third.

8. In view of these observations the authors believe that the cotton rat is the best experimental animal yet known for the production and study of tooth decay. The ease of production and the high, consistent incidence of carious lesions make possible many phases of experimental approach to the study of tooth decay, its causes and its control.

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# THE RELATION OF THE BLOOD LEVEL OF ASCORBIC ACID TO THE TISSUE CONCENTRATIONS OF THIS VITAMIN AND TO THE HISTOLOGY OF THE INCISOR TEETH IN THE GUINEA PIG<sup>1</sup>

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## FIVE FIGURES

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Information concerning the relation of the blood level of vitamin C to the well-being of the individual is of considerable interest. Such information should involve the relation of the blood level to the concentration of the vitamin in the other tissues of the body and to the presence or absence of any pathological changes. With such knowledge one could make an appraisal of the nutritional status, with respect to vitamin C, of an animal or of a human subject, by determining the vitamin C content of the blood. In this paper, we are reporting the results of a study of these relationships in guinea pigs upon different levels of intake of vitamin C.

## EXPERIMENTAL PROCEDURE

Sixty-six guinea pigs, thirty-three males and thirty-three females, of market stock with initial weights ranging from 188 to 517 gm. were maintained for 10 days on rabbit chow<sup>3</sup> plus cabbage ad libitum. This was to bring their body stores of vitamin C to approximately the same level. At this time they were transferred to a basal diet composed of:

Groond rolled oats (Quaker)	40 parts
Lactogen <sup>4</sup>	40 parts
Alfalfa meal	14 parts
Dried irradiated brewer's yeast <sup>5</sup>	5 parts
Sodium chloride	1 part
	<hr/> 100 parts

<sup>1</sup> Some of the data reported in this paper are taken from a dissertation submitted by Carl A. Kuether to the Graduate Council, George Washington University, April 1943, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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<sup>3</sup> Maritime Milling Co.

<sup>4</sup> Spray dried whole milk modified by the addition of milk fat and milk sugar. We wish to thank Nestle's Milk Products, Inc., for a generous supply of Lactogen.

<sup>5</sup> Fleischmann, Std. 360.



Supplemented with ascorbic acid, this diet produced good growth and prevented any obvious signs of deficiency in the guinea pig (Roe, Hall and Dyer, '41). Analysis showed this diet to contain 28 mg. of ascorbic acid per kilogram. The animals were fed this diet *ad libitum* after the following amounts of crystalline ascorbic acid<sup>6</sup> had been added per kilo of diet: 0, 50, 100, 150, 200, 300, 400, and 600 mg. A group of animals was placed on each level of ascorbic acid and one group was fed approximately 50 gm. of cabbage daily in addition to the basal diet. The animals were kept individually in cages with raised wire screen bottoms. Food consumption and weights were recorded daily. Ascorbic acid intakes were calculated from the food intakes.

Careful consideration was given to the problem of placing the animals on the test diets for a period that would produce a fairly constant level of ascorbic acid in the tissues for a significant time. Preliminary experiments showed that the tissue ascorbic acid content remained practically constant after the animals had been on a diet of the same ascorbic acid concentration for a period of 3 weeks. For example, the vitamin C concentration in the livers of guinea pigs taken off the preparatory diet and maintained at a constant low intake of vitamin C were 5.48, 4.24, 4.18 and 4.21 mg. per 100 gm. at 2, 3, 6, and 7 weeks, respectively, and the corresponding concentrations in the spleen were 17.7, 10.7, 11.5 and 10.4 mg. per 100 gm. It was decided to follow the more normal feeding procedure of incorporating the vitamin in the diet, so the animal would receive the supplement no faster than food is taken, rather than to give it in peak loads as when administered in solution by pipette or stomach tube.

After the animals had been on the diet for appropriate periods they were anaesthetized by an intraperitoneal injection of approximately 30 mg. of Nembutal per kilogram of body weight. A blood sample was collected by heart puncture and the tissues were rapidly removed for analysis. The tissues analyzed were: whole blood, liver, spleen, kidney, adrenal glands, skeletal muscle (gastrocnemius), cardiac muscle and brain. No animal was sacrificed until after having been on the test diet for at least 21 days. The average time for the groups of animals upon each of the test diets ranged from 26 to 38 days except one group, no. 8 of table 1, which was kept on the same level of vitamin intake for 73 days. The concentrations of ascorbic acid in the tissues of the latter group fall quite consistently in line with the levels in the tissues of the animals in the 26- to 38-day test periods. This similarity of results of analyses in the very long test period is further evidence that the 26- to

<sup>6</sup> We wish to thank Merek and Co. for supplying the ascorbic acid.

33-day periods were long enough to produce characteristic physiological or pathological findings in the animals.

Whole blood analyses were made by the method of Roe and Kuether ('43). Tissue analyses were made on a filtrate prepared by grinding the tissue to a uniform suspension with sand and 4% trichloroacetic acid. This was treated with Norit and filtered through a dry filter. The filtrate was analyzed as described by Roe and Kuether for blood filtrates.

The carcasses were examined grossly for the presence of hemorrhage, particularly around the knee joints and in the stomach, enlargement of the joints and beading of the costochondral junctions. The jawbones were removed and preserved in 10% neutral formalin for sectioning and microscopic examination of the incisor teeth.

## RESULTS

### *Chemical findings*

The results of the tissue analyses are summarized in table 1. Ascorbic acid intakes were calculated from the mean daily food consumption and body weight for the 14 days immediately preceding analysis. Increasing the level of ascorbic acid intake increased the ascorbic acid concentration in the blood and all the tissues analyzed, although not to the same extent. In going from the lowest to the highest intake the concentration of ascorbic acid in the whole blood increased about 6 times, the corresponding increases for the other tissues were: brain 6, muscle 11, kidney 14, spleen 15, heart and liver 19 and the adrenals 24 times. Thus there is considerable variation in the ability of the tissues studied to store vitamin C. From the values for the heart and skeletal muscle it is apparent that the concentration in cardiac muscle averages approximately  $2\frac{1}{2}$  times the concentration in skeletal muscle. Histologically and functionally cardiac muscle differs from skeletal muscle and it is not surprising to find this difference in ascorbic acid content.

It is questionable whether the tissue concentrations reported here represent the maximum concentrations attainable since higher values than these have been reported. Roe, Hall and Dyer ('41) published figures showing tissue concentrations per 100 gm. to be as high as the following: liver, 37 mg.; spleen, 42 mg.; brain, 24.5 mg.; and adrenal gland, 336 mg. These values were found in guinea pigs fed rabbit chow and cabbage. The maximum values found in the present study were: liver, 32.8; spleen, 49.1; kidney, 11.6; adrenal, 166; skeletal muscle, 3.12; brain, 22.8; heart, 8.98; and whole blood, 1.16 mg.%. With higher intakes of ascorbic acid it appears possible to raise the tissue concentrations to higher levels. In the group of animals with the highest meas-

TABLE 1

*Tissue ascorbic acid concentrations in guinea pigs upon different ascorbic acid intakes. All tissue concentrations are expressed in milligram per cent.*

GROUP	INTAKE <sup>1</sup>	NO. ANI- MALS	DAYS <sup>2</sup> ON DIET	WHOLE BLOOD	LIVER	SPLEEN	KIDNEY	ADRENAL	MUSCLE	BRAIN	HEART
1	.179 ± .027 <sup>3</sup>	9	26	.088 ± 0.19	.79 ± .13	2.25 ± .52	.55 ± .13	4.52 ± .90	.20 ± .03	3.05 ± 1.04	.29 ± .04
2	.532 ± .047	8	32	.139 ± .094	2.46 ± 1.03	7.98 ± 3.09	1.65 ± .69	16.78 ± 7.88	.52 ± .26	5.55 ± 1.57	1.09 ± .48
3	.701 ± .033	5	38	.143 ± .032	3.75 ± 1.13	12.76 ± 2.75	2.48 ± .43	28.52 ± 6.55	.76 ± .14	8.35 ± 1.95	2.16 ± .24
4	.900 ± .062	6	26	.193 ± .019	4.93 ± 1.04	17.05 ± 2.75	3.10 ± .46	37.73 ± 11.36	.86 ± .14	10.47 ± 2.77	2.56 ± .37
5	1.23 ± .12	7	31	.256 ± .043	7.68 ± 1.66	20.88 ± 3.16	4.35 ± .62	53.2 ± 10.6	1.13 ± .16	13.2 ± 1.5	3.35 ± .61
6	1.53 ± .09	7	35	.276 ± .065	9.71 ± 2.87	25.19 ± 4.60	5.14 ± 1.03	59.3 ± 15.0	1.34 ± .42	14.6 ± 2.1	3.83 ± .75
7	1.83 ± .08	7	34	.355 ± .082	11.59 ± 3.55	25.99 ± 4.40	5.64 ± 1.50	73.9 ± 15.8	1.63 ± .27	15.2 ± 2.2	4.59 ± .90
8	3.44 ± .72	9	73	.538 ± .192	15.45 ± 3.94	33.05 ± 6.86	7.87 ± 1.83	110.5 ± 27.9	2.24 ± .30	18.4 ± 3.0	5.49 ± .16
9	Cabbage	8	37	.754 ± .212	23.77 ± 6.02	42.83 ± 1.79	9.14 ± 1.68	118.6 ± 26.3	2.39 ± .42	18.6 ± 1.3	7.54 ± 1.29

<sup>1</sup> Milligram of ascorbic acid per 100 gm. body weight per day.

<sup>2</sup> Number of days the animals were on this intake before analysis (mean).

<sup>3</sup> Standard deviation of mean.

ured intake, namely 3.44 mg. per 100 gm. of body weight per day, the tissues had not yet reached saturation, since the group receiving cabbage had higher levels.

The ratios of the tissue concentrations of ascorbic acid to the whole blood concentration are shown in figure 1. With each tissue the ratio increases with increasing whole blood concentration to a maximum value. Increasing the whole blood concentration beyond this point resulted in a decrease in the ratio for spleen, kidney, brain, adrenal gland, heart and skeletal muscle, and no further increase in liver. The increasing ratio shows that the tissue concentrations of ascorbic acid are increasing at a faster rate than the whole blood concentration, indicating an active storage of vitamin C by the tissues. In every tissue studied the ratio reaches a maximum value at, or near, a whole blood concentration of 0.25 mg. %. This finding means that the tissues withdraw ascorbic acid from the blood most efficiently when the blood level reaches a concentration of 0.25 mg. per 100 ml. of whole blood. A higher blood level would have an advantage, however, in that it would bring about a greater storage of the vitamin for an emergency. The importance of these data is that they show the whole blood level of ascorbic acid reliably reflects the concentration of the vitamin in the tissues, hence a whole blood determination of ascorbic acid may be accepted as a significant indication of the nutritional status of the animal with respect to this vitamin.

From table 1, it will be seen that to maintain a whole blood concentration of 0.25 mg. per 100 ml., an intake of approximately 1.23 mg. of ascorbic acid per 100 gm. of body weight per day is necessary. If this rate of intake were transferred to man on the basis of body weight, the intake of a 70 kg. man would be 861 mg. per day. Translating upon a basis of body surface, the corresponding intake for man is calculated as follows. Using Meeh's formula for calculating surface area (Meeh, 1879; Lusk, '28, p. 123), the surface area of a 400 gm. guinea pig is 4.61 sq. dm. This makes the guinea pig requirement 1.07 mg. per sq. dm. per day when the whole blood level is maintained at 0.25 mg. per 100 ml. For an average man, height 5 ft. 8 in., weight 70 kg., the surface area (DuBois and DuBois, '16) is 1.83 sq. m. or 183 sq. dm. An intake equivalent to 1.07 mg. per sq. dm. per day would be 196 mg. for a man of 183 sq. dm. surface. Thus these studies indicate, by either a body weight or surface area translation of data, that the vitamin C requirement of the guinea pig is much greater than the requirement of man.

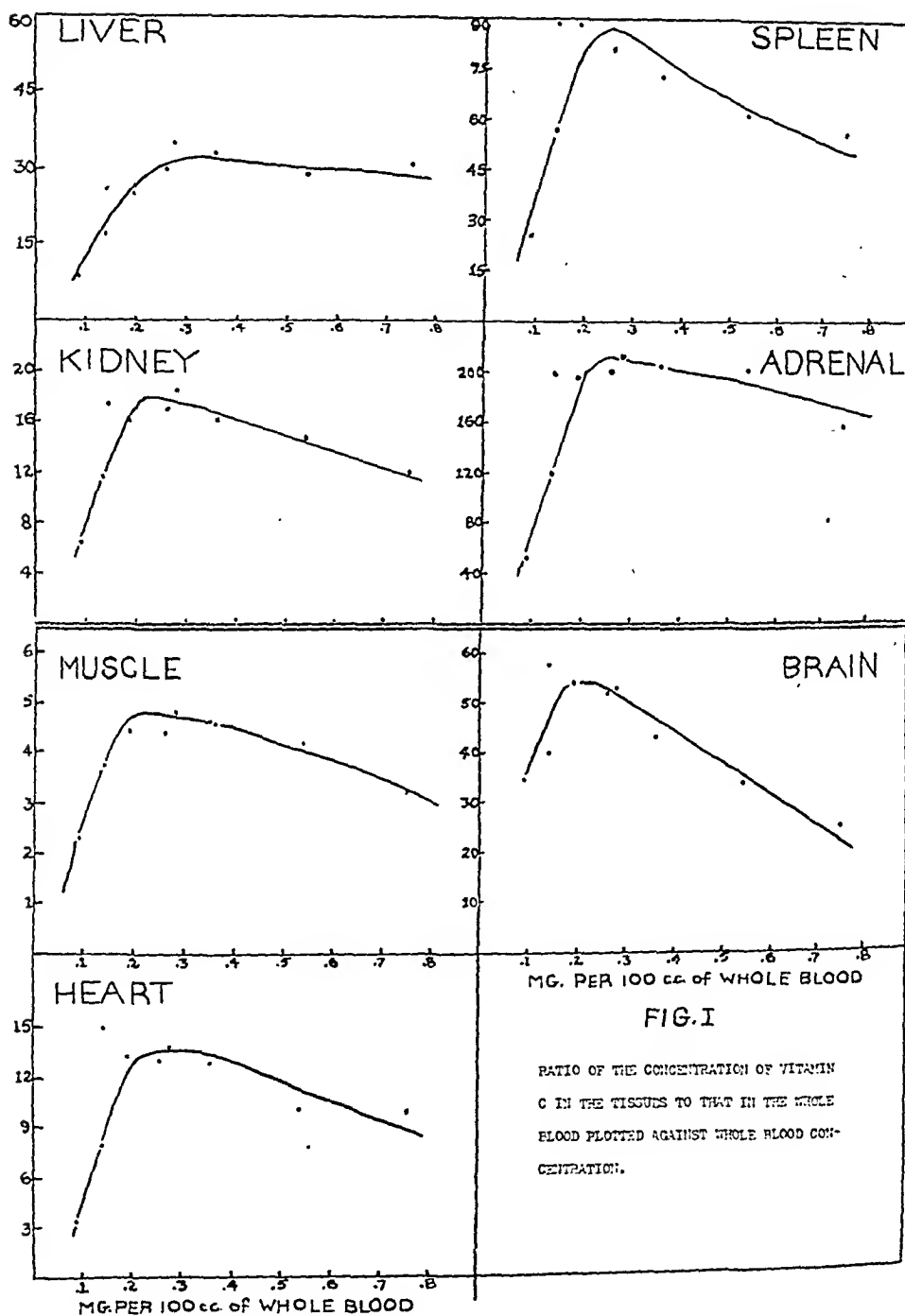


FIG. I

RATIO OF THE CONCENTRATION OF VITAMIN C IN THE TISSUES TO THAT IN THE WHOLE BLOOD PLOTTED AGAINST WHOLE BLOOD CONCENTRATION.

### *Histological findings*

The observations of Zilva and Wells ('19) revealed that the teeth were the first of the body tissues of the guinea pig to show an abnormality during the development of scurvy. Other histological work on the lesions in teeth of scorbutic guinea pigs has been done by Toverud ('23), Höjer and Westin ('25), Goettsch and Key ('28), Key and Elphick ('31), Fish and Harris ('34) and Boyle and his associates ('38, '40).

For the histological studies, fourteen male and twelve female guinea pigs were selected from the nine groups mentioned above. Each mandible with all teeth intact was fixed in 10% neutral formalin, decalcified in an alcoholic 5%  $\text{HNO}_3$  solution, embedded in nitrocellulose, sectioned longitudinally and stained with hematoxylin and eosin, and Mallory's triple connective tissue stain. These sections were then carefully studied for the presence of any lesions resulting from the dietary regimen of each group. The specific areas subjected to critical study were predentine layer, dentine with odontoblastic layer, enamel with ameloblastic layer, pulp cavity, cementum, periodontal membrane and the alveolar bone.

The use of longitudinal sections of the incisor teeth instead of cross sections, as are commonly used (Höjer and Westin, '25; Höjer, '26; Goettsch and Key, '28; Key and Elphick, '31; Dann and Cowgill, '35), has several advantages. Attention is called to the fact that each cross sectional level of the continually growing incisor tooth of the guinea pig has a different histological picture. The odontoblasts and ameloblasts alter their cytological structure and show senile changes as they migrate to the incisoral end of the tooth. Thus cross sections at different levels have a different histological appearance. Any comparisons of such cross sections could, therefore, lead to erroneous conclusions. Furthermore, longitudinal sections record the entire history of any recent acute scurvy. Changes in the incisor teeth, occurring early in the course of the deficiency, will still be observable at a later date in the sensitive cell layers which have subsequently migrated nearer the incisoral end of the tooth.

We studied incisor teeth rather than molar teeth (Fish and Harris, '34) because in the guinea pig the incisors grow, erupt and calcify continually throughout life. They show, therefore, in their longitudinal sections, the complete cycle of an acute ascorbic acid deficiency. The molar teeth were of little value in our experiments because their growth is largely limited to the early periods of life and hence are not sensitive

to the recent acute manifestations of the disease. Since the dentine and enamel layers in the molars of the guinea pig are of limited growth, the critical cell layers, the odontoblasts and ameloblasts, are largely dormant, inactive, senile cells and not sensitive indicators of mild scorbutic conditions.

There are two separate pathological changes occurring in the incisor teeth of guinea pigs upon a diet deficient in ascorbic acid. The first change is an upset in the calcification processes of the tooth. This is seen in several of the layers. The predentine layer becomes calcified. Normally this layer is comparatively free of calcium. The dentine often shows a mottling effect accompanied by areas or zones of decalcification. The pulp cavity shows spotty areas of irregular foci of calcification. The bone sections show some decalcification or faulty calcification of the alveolar bone. The cementum in animals on low vitamin C intake is largely replaced by a calcified matrix with entrapped cementoblasts.

The second constant change is a degeneration in the parenchymal cells of the teeth. The odontoblasts are the most sensitive of the parenchymal cells to a vitamin C deficiency (figs. 3, 4 and 5). In early ascorbic acid deficiency these cells become disorganized, vacuolated and show early signs of degeneration (fig. 3). With ascorbic acid blood levels below 0.15 mg. per 100 ml. these cells are completely degenerated (figs. 4 and 5). The ameloblasts are less sensitive to the lower levels of ascorbic acid intake (fig. 4) and show degeneration only in the most severe scurvy. The other cells—the cementoblasts, osteoblasts, and fibroblasts—show premature atrophy, loss of normal function and general disintegration.

These studies show clearly that the tooth is a good biological indicator of the nutritional status of the guinea pig in respect to vitamin C. The use of the teeth in guinea pigs is a much more accurate and sensitive means of detecting early scurvy symptoms than the usual methods. The presence of hemorrhages, especially around the knee joint, has been widely used as an indicator of scurvy. The appearance of such hemorrhages, however, is a gross manifestation of a severe deficiency and is of no value in the detection of mild scurvy. Our results show that tooth lesions appear at much higher blood levels of ascorbic acid than do the signs of hemorrhages (table 2). The retardation of growth has long been used as an indicator of ascorbic acid deficiency. Table 2 shows that growth still occurred in animals held for 4 to 5 weeks on a diet low in ascorbic acid. The odontoblasts, however, indicated varying degrees of the deficiency which were not shown by growth observations. Thus, the incisor teeth of the guinea pig, and more specifically the

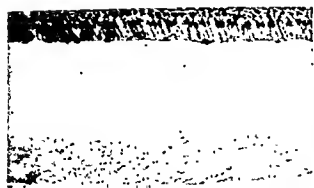


FIG. 2

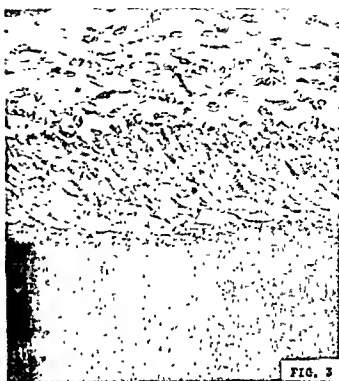


FIG. 3



FIG. 4

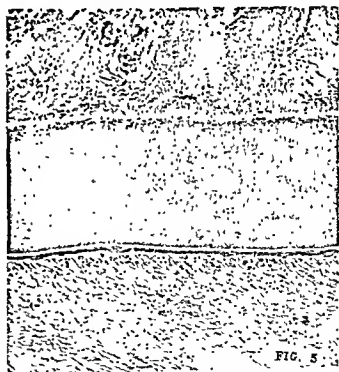


FIG. 5

Fig. 2 Guinea pig no. 163 — 0.42 mg. of ascorbic acid per 100 ml. of blood. Shows normal dentine, odontoblasts, predentine, enamel space and ameloblasts. Mag. 146 X.

Fig. 3 Guinea pig no. 148 — 0.15 mg. of ascorbic acid per 100 ml. of blood. Shows disorganization of odontoblastic layer, dilation and confluence of Tome's canals. Mag. 305 X.

Fig. 4 Guinea pig no. 144 — 0.08 mg. of ascorbic acid per 100 ml. of blood. Shows persistence of ameloblasts but disappearance of odontoblasts. Note the narrowing of the dentine layer. Mag. 146 X.

Fig. 5 Guinea pig no. 144 — 0.08 mg. of ascorbic acid per 100 ml. of blood. Shows degeneration of odontoblasts and disorganization of periodontal membrane. Mag. 146 X.



parenchymal cells of these teeth, offer a sensitive measure of ascorbic acid deficiency.

The present studies confirm the observations of other investigators (Zilva and Wells, '19; Toverud, '23; Höjer and Westin, '25; Key and Elphich, '31; Dann and Cowgill, '35; Boyle, Bessey and Howe, '40),

TABLE 2

*The relation of the blood level of ascorbic acid to the nutritional state of the guinea pig.*

GUINEA PIG NUMBER AND SEX	MG. ASCORBIC ACID PER 100 ML. OF WHOLE BLOOD	DEGREE OF TOOTH PATHOLOGY	HEMORRHAGE IN KNEE JOINT	WEIGHT GAIN GM./DAY <sup>1</sup>				
142	F	0.06	+	+	+	+	Marked	3.1
132	F	0.07	+	+	+	+	Marked	1.5
144	M	0.08	+	+	+	+	Slight	0.0
137	F	0.12	+	+	+		Slight	- 2.1
173	M	0.14	+	+	+		Slight	4.7
148	F	0.15	+	+			None	2.6
151	M	0.15	+	+			None	3.9
171	M	0.16	+	+	+	+	None	6.4
183	M	0.18	+				Slight	5.1
174	F	0.19	+	+			None	5.8
185	M	0.19	+	+			None	4.0
184	F	0.20	+				None	5.6
191	M	0.21	+				None	0.4
199	M	0.22	Normal				None	3.7
187	M	0.22	Normal				None	2.3
201	M	0.28	Normal				None	2.9
156	F	0.28	Normal				None	0.1
200	F	0.32	Normal				None	3.8
206	F	0.34	Normal				None	2.4
202	F	0.37	Normal				None	4.4
163	M	0.42	Normal				None	0.3
161	M	0.43	Normal				None	3.7
162	F	0.46	Normal				None	2.3
165	M	0.73	Normal				None	3.4
124 <sup>2</sup>	F	0.77	+				None	- 0.9
167	M	1.16	Normal				None	5.1

<sup>1</sup> For last 14 days.

<sup>2</sup> This animal was sick, refused food and lost weight.

which have shown that, of the various tissues of the body of the guinea pig, the incisor teeth are the most sensitive to a vitamin C deficiency. In addition we have established the blood and tissue ascorbic acid concentrations which result in a normal histological picture, and the levels at which pathological lesions occur. In table 2, a whole blood concentration of 0.22 mg. of ascorbic acid per 100 ml. represents a definite border-line where the earliest pathological signs of a deficiency occur

in the incisor teeth of guinea pigs. This level was determined by a histological diagnosis taking into consideration all of the criteria discussed above. The diagnoses were made by one of us (I.R.T.) without knowledge of the blood concentrations of ascorbic acid. In view of the rather narrow range of blood concentrations (0.08 mg. to 0.22 mg. of ascorbic acid per 100 ml.) above which normal histology was observed and below which the most severe pathological changes were found, table 2 shows a striking correlation between chemical findings and histological observations.

#### SUMMARY

1. A study of the relation of the blood concentration of ascorbic acid to the tissue concentrations and to the histology of the teeth has been made in the guinea pig.

2. The most efficient rate of withdrawal of ascorbic acid from the blood by liver, spleen, kidney, adrenal gland, brain, skeletal muscle and cardiac muscle occurs at a whole blood concentration of this vitamin near 0.25 mg. per 100 ml.

3. A concentration of ascorbic acid in the tissues reflected by a blood level above 0.22 mg. per 100 ml. of whole blood will prevent the appearance of any pathological lesions in the incisor teeth of the guinea pig.

4. The most obvious pathological changes due to ascorbic acid deficiency are to be found in the odontoblastic layer.

5. An alteration in the calcification pattern of the incisor teeth occurs at about the same blood levels as those associated with beginning changes in the odontoblastic layer, but the former changes are not as consistent or as easily interpreted.

6. Conventional methods of determining the presence of scurvy, such as the appearance of hemorrhages, reduced growth rate, or loosening of the teeth, are too inaccurate and insensitive to be of any value in detecting a mild ascorbic acid deficiency in the guinea pig.

7. An intake of 1.23 mg. of ascorbic acid per 100 gm. of body weight is required in the guinea pig to produce a whole blood level of 0.25 mg. per 100 ml. Translated to man, this requirement would be equivalent to an intake of 861 mg. per day on a basis of body weight or 196 mg. per day on a surface area basis. These data indicate that the vitamin C requirement of the guinea pig is higher than that of man.

8. Since there has been observed in the guinea pig a definite relation between the concentration of ascorbic acid in the tissues and that in the blood, it is suggested that the determination of the ascorbic acid content of the whole blood is the best procedure for evaluating the nutritional status of an animal with respect to vitamin C.

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# THE EFFECT OF FEED ON THE CRITICAL TEMPERATURE FOR THE ALBINO RAT<sup>1</sup>

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## ONE FIGURE

(Received for publication June 15, 1944)

Results reported in a previous publication on this subject (Black and Swift, '43) failed to verify the prevailing understanding that the critical temperature for an animal receiving food is lower than for the same animal during fasting. In this study the curve of heat production in relation to the environmental temperature for rats receiving feed was found to be similar to the one obtained earlier (Swift and Forbes, '39) for fasting rats except that somewhat more heat was produced in the feeding periods at temperatures above 26°C. The authors, however, were careful to point out that failure to find any effect of food on the critical temperature did not prove that such an effect is non-existent.

The problem has recently been subjected to further investigation with three improvements in technique, namely; (1) the heat production of the same six rats was determined during feeding and during fast, whereas in the previous studies different groups of rats were used for these two conditions; (2) the experimental subjects were reared at a practically constant environmental temperature of 30°C., which was within the zone of thermal neutrality, whereas in the earlier work they were reared at a slightly subcritical temperature; and (3) the diet fed was devised in the light of results obtained in other experiments at this laboratory (Forbes and Swift, '44) in a manner calculated to produce a maximum dynamic effect.

Six mature, male, albino rats were used in this study, which extended over a period of 3½ months. The ration consisted of a mixture of the stock colony diet<sup>2</sup> (56.2%) and ether-extracted beef muscle (43.8%). The ration contained 50.1% protein, and was characterized by an energy value of 4558 cal. per gram. The rats were fed nearly the maximum

<sup>1</sup> Authorized for publication on June 13, 1944 as paper no. 1238 in the Journal Series of the Pennsylvania Agricultural Experiment Station.

<sup>2</sup> Purina dog chow.

quantity they would consume, five of them each eating 14 gm. of feed per day, and the sixth animal consuming 11 gm. The daily allotment of feed was weighed out in two equal portions and fed at 7 A.M. and 5 P.M. The feed intake was constant throughout the entire experiment, except that preceding the fasting periods, which were always at least 8 days apart, two consecutive portions of feed were withheld. Thus, a rat entering the respiration chamber at 8 A.M., in a fasting experiment, would have consumed its most recent portion of feed, about 24 hours earlier.

The apparatus and procedure were similar to those which have been described in previous publications (Swift and Forbes, '39; Black and Swift, '43). Respiration measurements began at about 8 A.M. and continued for 8 hours. During this interval the  $\text{CO}_2$  was determined at the end of the first, second, third, sixth, and eighth hours. The entire period was used to determine the respiratory quotient and total oxygen consumption, while for the computation of the metabolism during rest, the subperiods of inappreciable activity and uniform  $\text{CO}_2$  production were used.

A distinct advantage of using the same six rats throughout the entire experiment was that, as the animals became accustomed to the routine, they remained quiet, not only while being weighed on a large chainomatic balance at the beginning and end of each respiration period, but also during a large part of the 8 hours spent in the respiration chamber. This is important, since the selection of the intervals of  $\text{CO}_2$  measurement to represent the metabolism during rest is fraught with some degree of uncertainty even when work adders are used as indicators of activity. In order to make such selection less arbitrary, in the experiment here reported, the intervals used to represent the metabolism during minimum activity were those in which the  $\text{CO}_2$  fell within  $\pm 6\%$  of the average for the day for the animal in question, excluding from the average a preliminary period since the animal was not exposed to the temperature of the respiration chamber until the beginning of this period. Furthermore, it is to be expected that there would be a lag in the physiological response of the animal to the environmental temperature.

The temperature of the respiration chamber, immersed in a water bath, was higher than that of the bath, especially at the lower temperatures. At a bath temperature of  $12.9^\circ\text{C}$ . the chamber registered  $15^\circ\text{C}$ ., whereas at a bath temperature of  $31^\circ\text{C}$ ., or above, the difference was negligible. The temperature inside the chamber was taken as the environmental temperature.

After the respiration measurements were completed, the urine and feces were collected separately during a feeding period of 8 days. The urinary nitrogen was used as a basis for the derivation of the heat produced by the oxidation of protein, and for the derivation of the non-protein respiratory quotient in the usual manner. In the fasting periods, the respiratory quotients were not corrected for the protein metabolism.

The chronological order in which the experiments were conducted is shown in table 1. In all cases, any feeding period was followed immediately by a fasting period at the same temperature. The live weight of the rats during the  $3\frac{1}{2}$  months was nearly constant, increasing from an average of 326 gm. to 336 gm. for the feeding periods, and from 300 gm. to 311 gm. during the fasting periods. The fasting heat production of each rat for each day was computed to a live weight of 310 gm. in accord with the 0.73 power of the live weight. The heat production of the feeding periods was used as determined.

The use of the same rats throughout the experiment allowed the application of Student's method in evaluating the results, the measurements of heat production as obtained from a given rat at two different temperatures constituting a pair of observations. The odds of significance shown in table 1 have been derived by comparing the values for the heat production of each of the six rats with the corresponding values at different temperatures. It is obvious that if the differences in environmental temperature are very small, no statistically significant difference could be shown to exist between the values for heat production at any two consecutive temperatures. The temperatures for which the heat productions were compared in evaluating the significance of the differences between these values, are indicated in table 1.

The respiratory quotients in all feeding and fasting periods were extremely uniform among the 6 rats for any given temperature. In accord with a finding by Swift ('32) that protein metabolism is unaffected by exposure to cold, it was considered that the protein metabolism in the feeding periods, at all temperatures, was represented without significant error by the urinary nitrogen obtained during the 8-day collection period, in which the environmental temperature was very close to 30°C. It is interesting to note that, throughout the range of thermal neutrality of the environment, about 71% of the total heat was derived from protein, while at 15°C. protein furnished 41% of the heat, with a concomitant increase in the amount of fat oxidized.

When this study was undertaken it was considered that the effect of feeding might be not only to lower the critical temperature, but that with fall in the temperature below the critical for fasting there would

TABLE 1  
Heat production of fed and fasting rats with change in environmental temperature.

ORDER OF EXPERIMENTS	TEMPERATURE	FEDING PERIODS						FASTING PERIODS			
		Avg. live weight	Avg. R.Q.	Avg. heat per hour	Odds of significance		Avg. live weight	Avg. R.Q.	Avg. heat per hour	Odds of significance	
					With reference to preceding temperature.	With reference to second preceding temperature				With reference to preceding temperature	With reference to second preceding temperature
9	15	gm. 340	.85	cal. 2501	.....	.....	gm. 318	.72	cal. 2392	.....	.....
8	23	340	.90	1677	10,000 to 1	.....	318	.74	1523	10,000 to 1	.....
7	26	342	.91	1531	275 to 1	10,000 to 1	315	.73	1233	3,332 to 1	10,000 to 1
6	.27	335	.91	1434	26 to 1	481 to 1	314	.74	1161	19 to 1	5,000 to 1
5	28	329	.91	1410	6 to 1	57 to 1	310	.74	1066	243 to 1	908 to 1
4	29	327	.92	1395	4 to 1	130 to 1	304	.74	1047	3 to 1	243 to 1
1	30	326	.91	1400	2 to 1	2 to 1	300	.74	1083	12 to 1	3 to 1
3	31	328	.92	1431	8 to 1	10 to 1	304	.76	1058	9 to 1	3 to 1
2	32	323	.93	1477	7 to 1	118 to 1	299	.75	1069	2 to 1	3 to 1
10	33	336	.93	1573	302 to 1	275 to 1	315	.77	1092	6 to 1	9 to 1
11	34	...	..	....	.....	....	311	.78	1187	45 to 1	454 to 1

be no increase in heat production during feeding periods until all of the heat increment of the food as observed during thermal neutrality had been utilized to prevent an increased metabolism due to cold. However, figure 1 shows that only about two thirds of the heat increment was effective in this respect, since the difference between the heat production of feed and of fast was about one third as great at subcritical temperatures as within the zone of thermal neutrality. In other words, we may consider that at subcritical temperatures, at least from 15° to 23°C., about two thirds of the heat increment is "useful" to the rat. It is recognized that any effect which feeding or fasting may have exerted on the constancy of body temperature is an integral part of the results obtained.

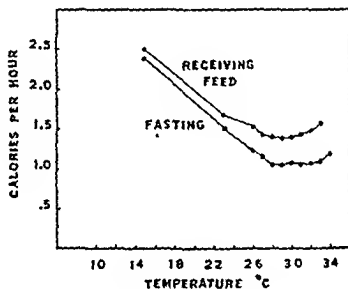


Figure 1

The results depicted in figure 1, and in table 1, are largely self-explanatory. The range of thermal neutrality for fasting rats is shown to be from 28° to 33°C. The observed critical temperature (28°C.) is 2 degrees lower than as reported in a previous publication (Swift and Forbes, '39), the upper limit of the zone of thermal neutrality being the same as previously found. It is possible that the residual effect of the very high protein diet fed in the present experimental work accounts for this difference. The range of thermal neutrality for the rats while receiving feed was from 27° to 32°C. Thus, the consumption of feed characterized by a high dynamic effect resulted in the lowering of both the upper and the lower limits of the zone of thermal neutrality by 1°C.

It is concluded, therefore, that energy metabolism studies with albino rats, either with or without feed, may properly be conducted at 30°C.



The plotting of the data for each rat produced a curve of heat production closely similar to the curve representing the six animals as shown in figure 1.

At the start of the experiment five of the six rats were 6 months of age, the sixth animal being slightly more than 1 year old. Each rat was fasted a total of eleven times, and in ten instances the oldest individual produced more heat (correlated to uniform live weight) at each temperature than did any of the younger rats. At all temperatures the heat production of the oldest rat exceeded the average heat production for the six rats. The amount by which the heat production of the oldest rat exceeded the average heat production of the other five rats in the eleven fasting periods was 12.9%. This is in accord with a result reported by Benedict and MacLeod ('29) who found that the metabolism of fasting rats increases with age.

#### SUMMARY

The heat production of six mature, male, albino rats was determined while receiving a diet characterized by a high dynamic effect, and also while fasting, at environmental temperatures ranging from 15° to 34°C. The zone of thermal neutrality for the fasting rats was found to be from 28° to 33°C., the effect of feed being to lower both limits of this range by 1°C. About one third of the heat increment of the food as observed in the zone of thermal neutrality was manifest below the critical temperature.

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# THE BIOLOGICAL ASSAY OF VITAMIN A BY MEANS OF ITS INFLUENCE ON THE CELLULAR CONTENTS OF THE VAGINA OF RATS

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## ONE FIGURE

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Evans and Bishop ('22) observed that young rats fed a vitamin A deficient diet failed to show the normal cyclic changes in the cellular contents of the vagina and the squamous cells normally present during estrus were seen continuously. Attempts were made by a number of workers, Coward ('29), Hohlweg and Dohru ('30), Bauman and Steenbock ('32), Moll et al. ('33), Coward et al. ('35), Coward ('38) and Goss and Guilbert ('39) to use this phenomenon as a criterion of response for the biological assay of vitamin A and its precursors.

In view of the relatively low degree of precision attained, duration of the test and the difficulties encountered in performing a satisfactory assay by the methods outlined in the United States Pharmacopocia XII ('42) and British Pharmacopocia ('32) addendum ('36) it appeared worthwhile to make a further study of the vaginal smear method to see if a practical and convenient procedure for the biological assay of vitamin A could be obtained yielding more precise values than those depending on an increase in body weight.

## METHOD

During a period of 10 to 12 days 100 or more female rats at 21 days of age are obtained from the stock colony. They are fed the maintenance diet (table 1) and kept in screened-bottom cages until vaginal introitus occurs in the whole group.<sup>1</sup> At this time the whole group is ovariectomized (Burn, '37), and 2 to 3 days following the operation vaginal smears are taken from the whole group. Depending on the dominant type of cell present in the smear the rats are retained on the maintenance diet or fed the vitamin A free diet (table 1). If leucocytes

<sup>1</sup> The mean age of vaginal introitus in the Laboratory of Hygiene's colony of rats of Wistar strain is  $45.2 \pm 4.1$  days.

predominate, the rats are fed the vitamin A free diet and the smears are examined bi-weekly until there is a dominance of squamous cells mixed with epithelial cells and leucocytes (stage 3; see below). Some of the rats reach this stage or one approaching it on the maintenance diet and they are retained on this diet until the remainder of the group shows the same evidence of depletion. When the whole group is near depletion, as shown by the vaginal smear, the rats are weighed, placed on the vitamin A free diet and smears are examined daily until all show uniformly a dominance of squamous cells (stages 1 and 2). This usually requires 3 to 4 days. The rats are weighed again and any showing a marked loss in weight (10 gm. or more) are excluded. The rats are divided into at least six groups on the basis of equal distribution of body weight. Three of the groups receive doses of the standard and

TABLE 1

*Diets used for the biological assay of vitamin A by the vaginal smear technique.*

COMPONENT	DIET	
	Vitamin A free	Maintenance
Rice starch, dextrinized	65%	58%
Casein, vitamin-free (Labco)	18%	18%
Brewers' yeast, fat-free	8%	..
Brewers' yeast	..	8%
Corn oil (Mazola)	5%	5%
Wheat germ	..	7%
Salt mixture (U.S.P.XII)	4%	4%
Vitamin D (viosterol): per 100 gm.	300 I.U.	300 I.U.

the remaining three groups doses of the sample. The doses of vitamin A are made up in cocoanut oil and administered orally morning and night for 2 successive days in a volume of 0.1 ml. by means of a tuberculin syringe bearing a 20-gauge needle cut off at 1.5 cm. In the case of low potency products the volume administered can be increased to 0.25 ml. and the dosing time extended to 4 days. Beginning on the day following the last dose vaginal smears are taken daily for 3 successive days and subsequent smears are made according to the type of cells present in the last smear.

A smear is made with pledgets of cotton on a tooth pick, which is moistened with water, introduced into the vagina, rotated once and laid on a slide bearing the number of the rat. A drop of water is added to the slide, the cotton rubbed through this and the smear read without staining under the low power of a microscope. The smears are recorded as indicated in table 2 depending on the predominant type of cells present.

All rats in stages 1 and 2 are regarded as depleted of vitamin A and the remainder as not depleted. As soon as the smears fall into stages 3 and 4, daily smears should be taken and examined so that the end-point of the depletion (stages 1 and 2) may not be missed. The appearance of the smears of the depleted rats is similar to the full estrus smear encountered with ovariectomized rats treated with estrogen or the squamous stage of the normal estrus cycle except a few leucocytes persist in some and stage 2 is included to take care of these.

TABLE 2

*Scale used in recording the character of the vaginal smear and the corresponding stage in vitamin A deficiency.*

STAGE	SYMBOL	TYPES OF CELLS	STAGE	SYMBOL	TYPES OF CELLS
1	+	Squamous cells	5	+ L - E	Leucocytes with a few epithelial cells
2	± S	Squamous cells with a few leucocytes	6	+ L - S	Leucocytes with a few squamous cells
3	+ S - E	Squamous cells with epithelial cells and a few leucocytes	7	-	Leucocytes
4	+ E - S	Epithelial cells with squamous cells and a few leucocytes			

The response is taken as the number of days, beginning with the first day of dosing, required for the cellular contents of the vagina to change from the squamous cells, stages 1 and 2 (depleted state) to leucocytes or a mixture of leucocytes, epithelial and squamous cells, stages 3 to 7 (curative state) and return to squamous cells, stages 1 or 2 (depleted state). As soon as any rat on a dosage level shows stages 1 or 2 (depleted state) it is returned to the maintenance diet. Three to 4 days after an assay is completed the whole group of rats is weighed, the smears examined and the animals given the vitamin A free diet. When the whole group is uniformly depleted (usually 3 to 5 days later) the rats are weighed again, grouped as above and utilized for another assay. The same group of rats can be used repeatedly in this manner for eight to ten assays or until it becomes too small to use to advantage.

#### EXPERIMENTAL

To evaluate the method using a relatively wide range of doses and to establish the relation between dose and response which gives the best measure of potency, the following experiment was performed.

Eighty-four rats were depleted of their vitamin A reserves as described above and assembled in six groups of fourteen rats each. Doses of vitamin A from 50 to 524 International Units (I.U.) were prepared from the Canadian Standard Reference oil by dilution with coconut oil, each dose being 1.6 times the previous dose. The results are shown in table 3 and graphically in figure 1. It is seen that the points fit a

TABLE 3  
*The response of rats to vitamin A.*

GROUP NO.	NO. OF RATS	DOSE IN I.U.	LOG DOSE IN I.U.	MEAN ARITH. RESPONSE IN DAYS	MEAN LOG RESPONSE IN DAYS	S.E. $\times 1.96^1$ LOG RESPONSE
1	14	50.0	1.6990	9.00	.9543	.0411
2	14	80.0	1.9031	11.49	1.0602	.0280
3	14	128.0	2.1072	14.94	1.1742	.0269
4	14	204.8	2.3113	18.86	1.2754	.0201
5	14	327.7	2.5155	24.47	1.3888	.0085
6	14	524.3	2.7195	30.47	1.4838	.0109

$$^1 \text{S. E.} = \text{Standard Error} = \sqrt{\frac{\sum d^2}{n(n-1)}}$$

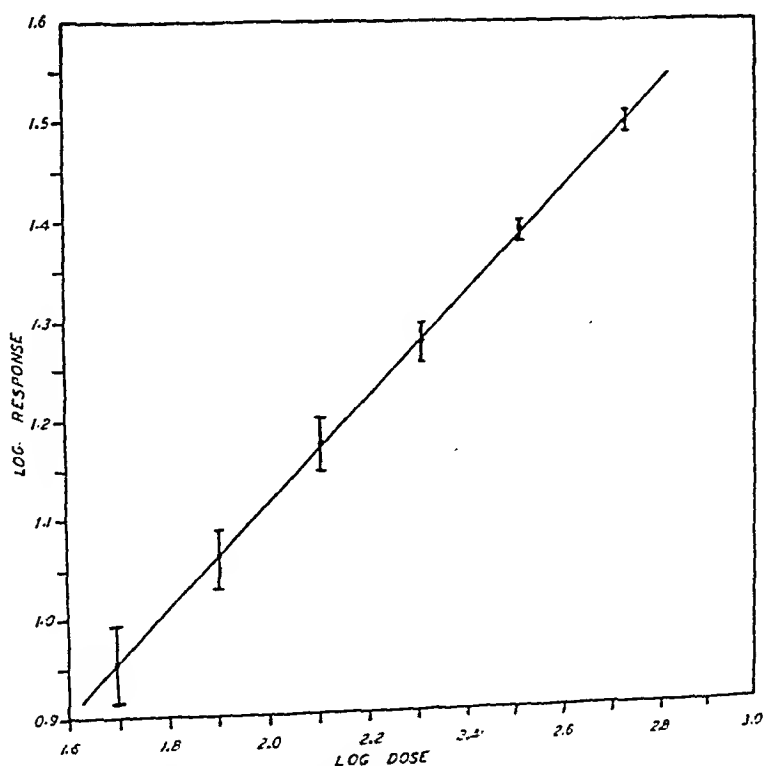


Fig. 1 The logarithmic relation between dose and response to vitamin A. The vertical lines above and below the points are  $1.96 \times$  the standard error of the means expressed as logarithms.

straight line reasonably well over this relatively wide range of doses, when the logarithm of the dose is plotted against the logarithm of response. The straight line is represented by the equation  $\log. y = 1.222 + 0.522 (\log. x - 2.209)$ .

The results were subjected to factorial analyses to determine the best method of expressing the values for a linear dosage response curve. Groups 1, 3 and 5 were taken as the standard and 2, 4 and 6 as the sample. Since most graded response assays follow a logarithmic dose and an arithmetic response relationship this was calculated first. According to the variance ratio (table 4, column 2) the dosage response curve shows a significant amount of curvature and the lines are not parallel. The critical value at the 5% level of significance and the number of degrees of freedom in this experiment is approximately 4.0 (Fisher and Yates, '38). On the other hand when the logarithm of the

TABLE 4

*The variance ratios using the logarithm of the dose and the arithmetic response and the logarithm of the dose and the logarithm of the response.*

	VARIANCE RATIO LOG DOSE — ARITH. RESPONSE	VARIANCE RATIO LOG DOSE — LOG RESPONSE
Sample	114.83	04.16
Slope	1326.09	1140.28
Parallelism	14.38	.18
Curvature	23.21	.07
Opposed curvature	.23	.00

dose and the logarithm of response are used, the variance ratio (table 4, column 3) indicates there is not a significant degree of curvature and the lines are parallel. The variance ratios were calculated in this manner for sixteen assays; and, using the logarithm of the dose and the arithmetic response, the ratio exceeded the critical value in eight cases with respect to curvature and in two cases with respect to parallelism, while using logarithm of the dose and logarithm of response the ratio was well below the critical value in all assays. From these results it is concluded that this type of assay follows a logarithm of the dose and a logarithm of response relationship.

Referring to the data in table 3 using groups 1, 3 and 5 as the standard and 2, 4 and 6 as the sample the most probable value for the ratio  $M$  was 1.55 with a range of 1.40 to 1.70 ( $p = 0.95$ ). The actual value was 1.60.

The precision and reproducibility of the method was determined by assaying the Canadian Standard Reference oil against itself. The oil or various dilutions of it were presented as unknowns to the authors. An approximate potency was revealed to permit the preparation of desir-

able dosage levels and the actual potency was reported after the assay and the calculations were completed. Three dosage levels of the standard and unknown in cocoanut oil were used in all these assays and the data were calculated by the method of Bliss and Marks ('39). The assays were set up with equal numbers of rats on each dosage level, but occasional deaths occurred and the whole group for the purpose of the calculations was reduced accordingly by striking off the last animal in the list where extra ones occurred in order to balance the groups. The results are shown in table 5. It is seen that the potency determined by assay agrees satisfactorily with the true potency.

TABLE 5  
*The precision and reproducibility of the method of assay.*

ASSAY	NO. OF RATS	POTENCY FOUND IN	LIMITS OF ERROR OF TEST $\times 1.96$	ACTUAL POTENCY
		<i>I.U.</i>	<i>%</i>	<i>I.U.</i>
1	42	1830	87.2 — 114.7	1700
2	54	1730	93.3 — 107.2	1700
3	60	1460	93.7 — 106.6	1445
4	48	780	88.2 — 113.3	790

In order to show the versatile application of the method the results obtained with a group of miscellaneous products are presented in table 6. These products were assayed against the Canadian Standard Reference oil using cocoanut oil as the diluent for the dosage levels except in the case of spinach, mixed vegetable juices, milk chocolate powder and dehydrated egg powder, where water was used. It was necessary to extend the dosing period to 3 days with the butter and the spinach and 4 days with the mixed vegetable juices and egg powder because of their low potency. The dosage levels of these latter products were made adequate by increasing the amounts administered, and the standard in oil was administered in the same manner. The slopes of the dosage response curves of the products in aqueous medium were not significantly different from that of the standard in oil. No difficulties were encountered in these assays and the method appears applicable to a variety of products.

To determine if variations in the initial weight of the rats influenced the precision of the assay, the body weights of all rats in each of four assays were reduced to a common value by analysing the data by covariance (Bliss and Marks, '39). The reduction in the error (table 7) is not sufficient to require a correction or to limit the ranges of body weight below those used in these tests. It is seen from the standard deviation of the mean body weight that considerable variation existed at the time of assembling the groups.

The same sample of cod liver oil was assayed three times against the International Standard  $\beta$  Carotene by (a) the vaginal smear method and (b) by the procedure outlined in the British Pharmacopoeia ('32) addendum ('36) using the 5 weeks dosing schedule. It is seen (table 8) that the vitamin A potency determined by the vaginal smear method is approximately the same as the potency determined by the increase in weight method. The difference between the two weighted means is not significant ( $t=0.526$ ). The three assays by the vaginal smear method

TABLE 6  
*Miscellaneous assays.*

PRODUCT	NO. OF RATS	POTENCY <i>I.U. /per gm.</i>	RANGE OF POTENCY SM X 1.96
Cod liver oil	48	1480	1300 to 1680
Shark liver oil	48	27700	24700 to 31000
Distilled vitamin A concentrate	72	307400	286500 to 329800
One per cent vitamin A acetate in Wesson oil	60	46000	41800 to 51300
Dairy butter	55	81	76 to 86
Strained spinach	68	59	52 to 67
Mixed vegetable juices	50	22	17 to 28
Milk chocolate powder	56	184	157 to 215
Dehydrated egg powder	33	7	4 to 11

TABLE 7  
*The effect of body weight on the variance for experimental error.*

ASSAY NO	NO. OF RATS	MEAN WT. GM.	STANDARD DEVIATION	VARIANCE FOR EXPERIMENTAL ERROR	VARIANCE FOR EXPERIMENTAL ERROR CORRECTED FOR BODY WEIGHT
1	84	136.3	18.3	.00228	.00222
2	48	188.9	16.4	.00257	.00257
3	54	180.5	18.9	.00134	.00129
4	60	171.2	21.1	.00126	.00124

were done on the same group of rats and it required approximately 2 weeks to complete each of them with an interval of 4 to 6 days between assays. On the other hand three groups of rats were required for the increase in weight method which involved a 5-weeks dosing schedule and the assay in each case was prolonged for approximately 7 weeks due to replacements on account of deaths during the first 2 weeks. It is quite evident from these results that the vaginal smear method shows a decided advantage with respect to precision and economy of material, time and effort.



Hickman et al. ('44) have shown that mixed natural tocopherols enhance the growth promoting properties of vitamin A. It was considered advisable to see if tocopherols produced a similar effect in the vaginal smear response to vitamin A. This was done by assaying the Canadian Standard Reference oil against itself and administering a tocopherol<sup>2</sup> or mixed natural tocopherol<sup>3</sup> with the doses at the various levels (total dose) indicated in table 9, assays 1 to 7. Also the International Standard  $\beta$ -Carotene was assayed against itself with the addition of a tocopherol to the sample (assay 8 table 9). It is seen that when a tocopherol is administered with both the standard and the sample in amounts of 0.5 mg., the ratio of the potency of the sample to that of the standard remains unchanged within the limits of error

TABLE 8

*A comparison of the vaginal smear method and the increase in weight method for the assay of vitamin A.*

ASSAY	NO. OF RATS	POTENCY IN I.U.	LIMITS OF ERROR SM $\times$ 1.96 IN PER CENT
Vaginal smear method			
1	60	1482	88.6 to 112.9
2	56	1561	86.0 to 113.6
3	57	1445	89.9 to 111.2
Weighted mean		1480	93.2 to 107.3
Increase in weight method			
1	52	1727	82.0 to 124.7
2	48	1165	68.9 to 145.2
3	52	1560	69.4 to 143.7
Weighted mean		1561	84.5 to 118.3

of the assay. There was no evidence from the variance ratios or the slope of the dosage response curve that the tocopherol produced any irregularities in this assay. On the other hand, when a tocopherol or mixed natural tocopherol was added to the doses regarded as the sample, there was a significant difference between the standard and sample in assays 2, 4 and 5. In all these assays the tocopherol shows the power to enhance the activity of the sample, and the mixed natural tocopherol produced a greater effect than the  $\alpha$  tocopherol. However, when the  $\alpha$  tocopherol was administered, adding 0.5 mg. to the standard and 2.0 mg. to the sample (assay 6), there was not a significant difference between standard and sample. This was repeated (assay 7) with 1.0 mg. of mixed natural tocopherol added to the standard and 4.0 mg.

<sup>2</sup> From the S. M. A. Corporation.

<sup>3</sup> From Distillation Products, Inc.

to the sample with the same result. These experiments indicate that if an oil is suspected of containing tocopherol the true vitamin A activity can be determined if an amount of tocopherol above the optimum (0.5 mg.) is administered with the standard and a similar amount added to the doses of the sample since an excess above 0.5 mg. does not produce an added effect. The experiment with  $\beta$  carotene illustrates that tocopherol enhances its activity since there is a significant difference between

TABLE 9

*The effect of tocopherols on the response to vitamin A.*

ASSAY NO.	NO. OF RATS	RATIO OF SAMPLE TO STANDARD	LIMITS OF ERROR IN PER CENT $\pm M \times 1.96$	VARIANCE RATIO FOR SAMPLES	REMARKS
1	54	1.02	93.5 to 107.1	0.276	Canadian Standard Reference oil with 0.5 mg. a tocopherol added to both standard and sample.
2	44	1.22	88.7 to 112.7	13.04	0.5 mg. a tocopherol added to sample.
3	28	1.20	79.0 to 126.5	2.83	0.5 mg. a tocopherol added to sample.
4	44	1.56	79.9 to 125.1	17.10	4.0 mg. mixed natural tocopherol added to sample.
5	44	1.62	77.0 to 129.8	19.40	1.0 mg. mixed natural tocopherol added to sample.
6	60	0.96	86.7 to 115.3	0.41	0.5 mg. a tocopherol added to standard and 2.0 mg. a tocopherol to sample.
7	44	1.01	88.0 to 113.6	0.11	1.0 mg. mixed natural tocopherol added to standard and 4.0 mg. mixed natural tocopherol to sample.
8	72	1.10	93.4 to 107.4	4.11	$\beta$ carotene with 0.5 mg. a tocopherol added to the sample.

the standard and sample according to the variance ratio. These experiments confirm those of Hickman et al. ('44) indicating that tocopherols augment the effect of vitamin A, but this augmentation can be controlled in an assay by ensuring that tocopherol is administered with the doses in amounts above the optimum to both standard and sample.

It was observed that the squamous cells in the vaginal smear of depleted rats disappeared 3 to 5 days after dosing with vitamin A. Attempts were made to use the absence of squamous cells from the smears as a criterion of response in the same manner as the absence of leucocytes is used as a criterion of response in the assays of estrogens. Rats

were depleted as described above and assembled for four dosage levels of vitamin A. The doses were administered orally once daily for 5 days and the smears were examined daily for 6 or more days after dosing. The percentage of rats on each dosage level showing the absence of squamous cells in their smears between the third and fifth day after dosing was taken and the slope of the dosage response curve calculated according to the method of Bliss ('35) for the quantal response data. It is seen (table 10) that the slope (b) of the dosage response curve is relatively low for a precise method of assay. Difficulties were encountered in obtaining clear-cut end points and this criterion of response was rejected in favor of the one described above.

TABLE 10  
*Quantal response assays for vitamin A.*

CURVE NO.	NO. OF RATS	b	vb	RD <sub>50</sub> I.U.
1	46	5.045	1.943	99.7
2	60	3.095	2.704	129.2
3	57	4.325	1.281	129.2
4	61	1.445	0.987	95.4
5	34	3.146	3.508	109.2
6	65	1.568	0.216	136.6
Mean		2.103		

### DISCUSSION

The assay of vitamin A by its effect on growth rate, as measured in terms of the weight of rats fed on a relatively purified diet deficient in vitamin A and undoubtedly in other factors as well, makes such an assay method unsatisfactory on account of the relatively non-specific type of response. While vitamin A has some effect on growth there are a number of factors concerned in such a response, and vitamin A is not necessarily the limiting one under the existing conditions. On the other hand the change produced in epithelial structures is one of the most characteristic effects of a vitamin A deficiency. It is completely reversible and occurs regardless of age. The role of vitamin A in the normal differentiation of cells has thus far not been explained satisfactorily. The production of keratinized cells in the cellular contents of the vagina of rats deficient in vitamin A makes a readily available and practical means of measuring the response to vitamin A.

The diet usually employed to produce a vitamin A deficiency is not well balanced with respect to the B vitamins; and to render the rats deficient in vitamin A but in an otherwise more satisfactory nutritional condition, a maintenance diet was employed for the primary depletion

period and to support the rats at low levels of vitamin A reserve between tests. A number of substitutions and additions to the A free ration were tried in an attempt to devise a satisfactory maintenance diet, namely (a) feeding the vitamin A free diet and a maintenance dose of vitamin A, (b) adding skim milk powder to the amount of 3% of the diet, (c) substituting unextracted commercial casein for extracted casein in the A free diet, (d) adding wheat germ to the A free diet to the amount of 5, 7 and 10%, and (e) adding wheat germ to the amount of 7% and substituting unextracted casein for extracted casein and brewers' yeast for fat-free yeast. The last alteration of the A free diet proved more satisfactory and convenient. When the maintenance diet (table 1) was prepared at weekly intervals in this manner and fed during the primary depletion period, a steady increase in weight occurred and the rats were in a better nutritional condition than when the A free diet alone was fed. The amount of carotene in the maintenance diet appears to be just enough for subsistence and at the same time to render the rats easily depleted upon feeding the A free diet. No difficulties have been encountered with different lots of commercial casein and wheat germ obtained over a period of 2 years.

Attempts were made to hasten vaginal introitus by the administration of estrogens. Following subcutaneous injection of an aqueous solution of diethylstilbestrol (0.2 ml. = 2  $\mu$ g.) into a group of 30 rats 25 to 35 days of age and estrone (0.2 ml. = 5  $\mu$ g.) into another group of 30 rats of the same age, rupture of the vaginal membrane occurred 4 to 6 days later and the rats were ready for ovariectomy. Although this treatment is unnecessary it has the advantage of saving time in checking the rats for vaginal introitus during the primary depletion period. In a few cases when puberty was allowed to proceed naturally, and vaginal introitus was delayed in a few rats in the group, the membrane over the vagina was punctured with a needle. This intervention and the administration of estrogens did not interfere with the subsequent reaction of the rats to vitamin A.

Intact female rats have been used by other workers to demonstrate the effect of vitamin A on the cellular contents of the vagina. It was considered that the ovariectomized rat would be a more satisfactory animal to use, since the removal of the ovaries ensures that the response will not be interpreted as a normal or a prolonged estrus effect. No post-operative infections occurred when the vitamin A deficient rats were ovariectomized without precautions as to sterility, and no instances of regeneration of ovarian tissue have been encountered. This operation is a relatively simple one requiring 3 to 4 minutes per rat,

and after one has obtained experience in administering the anaesthetic the deaths at operation are nil.

The progress of the depletion and the response is quite adequately followed by the system of symbols recommended. In some cases the smears are slightly difficult to interpret due to the persistence of leucocytes. This usually occurs when the rats are suffering from an infection of the urogenital or respiratory tracts. A confirmatory reading often clears up any doubts regarding the end point of the test. It was found that as soon as the cells in the smears become predominately squamous in the vitamin A depleted rats, such a condition persists until death occurs. The smears are examined only once daily, since it was considered impractical and inconvenient to read them more frequently. Smears may be read quite satisfactorily 24 hours after they are obtained providing they are moistened and covered. This practice of having an assistant take the smears on Sunday and to delay the reading until Monday was followed in a few assays.

The response of the rats on the low dosage level was questionable in a few assays because the dose of vitamin A was not sufficient to cause a change from the squamous cells to epithelial cells and leucocytes during the 3 days after dosing. Groups of rats reacting in this manner were excluded from the calculations. It has been observed that the dosage levels can be arranged for unknown oils quite satisfactorily when the L value per gram as determined colorimetrically by the method of Dann and Evelyn ('38) is interpreted directly in International Units. The minimum total dose for a satisfactory response in our colony of rats is between 45 and 60 I.U. of vitamin A.

Greater uniformity is obtained if complete groups of rats are assembled for a test, rather than to begin dosing a few rats at a time as they become depleted. The former method requires some shifting of the rats from the A free diet to the maintenance diet and vice versa, but it is only in the primary depletion period that this shifting requires much attention. After the first assay is completed the rats become depleted at a remarkably uniform rate for subsequent assays.

A linear dosage response relationship is obtained by plotting the logarithm of the dose against the logarithm of the response and in this respect the method differs from most assay procedures of a continuous variate type in which a linear relationship is obtained by plotting the logarithm of the dose against the arithmetic response. In this connection De Graff et al. ('41) have shown that the assay of digitalis glucosides by the embryonic chick heart follows a logarithm of the dose and a logarithm of the response relationship. To show that the varia-

tions in response of the individual dosage levels follow a normal distribution curve, the response obtained with each of three dosage levels of the Canadian Standard Reference oil were pooled for eleven assays which gave 100 rats per dosage level. When the days were grouped for each dosage level and the resulting percentages calculated, a normal curve of distribution for each dosage level was obtained.

The precision of this method of assay is relatively greater than that obtained with the increase in weight method of assay. Typical values of the limits of error attainable by the method using different numbers of rats per assay are shown in tables 5 and 6 and a direct comparison with the increase in weight method is shown in table 8. From these results an error of  $\pm 12$  to 15% using 60 rats per assay and  $p$  at 0.95 is quite readily attained. Most of the assays reported here have been calculated by the method of variance and covariance of Bliss and Marks ('39) to show that they fulfill the requisites of a satisfactory method of biological assay. This method of calculation requires the dosage levels to be adjusted at equally spaced intervals and that equal numbers of rats be used for each level. These requirements are not readily obtainable in routine assays due to deaths of a few rats from respiratory infections during the course of an assay and the adjustment of the dosage level of low potency products. The method of calculating biological assays of a continuous variate type proposed by Irwin ('37), in which a system of weighting for numbers of rats on each dosage level is used, was employed for a few assays that did not meet the above requirements and in order to check the results obtained by the method of Bliss and Marks ('39).

The assays reported in table 6 show the method to be applicable to a variety of products. To attain desirable dosage levels with some of the products it was necessary to continue the dosing period for more than 2 days. There was no evidence that this alteration in technique produced results different from the dosing period of 2 days recommended for fish oils. The slopes of the dosage response curves obtained for products in aqueous medium were not significantly different from the slopes obtained for the standard in an oily vehicle.

The reduction in the error of the method obtained by correcting for body weight does not appear to be significant from the results obtained in the assays in table 7. In all the assays conducted the rats have been assembled on the basis of equal distribution of body weight in each group, and variations in weight within the groups on an assay have been as much as 50 gm. It appears from the results obtained that the response is independent of body weight within these limits. Although

considerable uniformity in depletion time during the primary depletion has been observed to occur within litters, no attempt has been made to pair off litter mates in the assays due to the impracticability of such a procedure.

The observation by Hickman et al. ('44) that vitamin E augments the activity of vitamin A has been confirmed. Most of the evidence favors the conclusion that the tocopherols act as anti-oxidants during the absorption of vitamin A from the gastro-intestinal tract. Hickman et al. ('44) have shown that the maximum effect is produced at approximately 0.5 mg. of tocopherol per rat per day. On this basis, a product containing vitamin A as well as vitamin E can be assayed quite satisfactorily by administering this amount of tocopherol with the dosage levels of vitamin A to both standard and sample. Tocopherol itself was shown to possess no vitamin A activity when administered to a group of 20 rats in amounts of 340 mg. per rat. It does not appear practicable to prevent errors arising from the tocopherol effect by the addition of tocopherol to the diet because it is readily oxidized in such a dietary medium; also the addition of a natural product containing vitamin E does not appear feasible because most of them contain significant amounts of carotene. Since tocopherols in products assayed for vitamin A yield an added biological effect, errors can quite conceivably occur in the determination of vitamin A activity by the spectrophotometric or chemical methods when the results are reported in terms of biological activity. Considerable evidence is available indicating that such methods do not give reliable estimations of the biological activity of the product due to the different forms in which vitamin A may exist, hence the necessity of a precise biological assay method for vitamin A.

It was disappointing to obtain the relatively low values for the slopes of the dosage response curves with the accompanying low degree of precision in the quantal response method of assay shown in table 10. This method had the advantage of being short, and convenient. Preliminary assays using other dosing times were tried without any success in improving the precision of the method.

#### SUMMARY

A method of biological assay of vitamin A and its precursors based on the changes produced in the cellular contents of the vagina of ovariectomized rats is described. The dosage response relationship was analysed by methods of variance and covariance and shown to be logarithmic. The method shows considerable advantage over the increase in weight method with respect to precision and economy of time,

material and effort. The enhancement of the biological activity of vitamin A by tocopherols has been confirmed and a method of overcoming this effect in the estimation of vitamin A activity is described.

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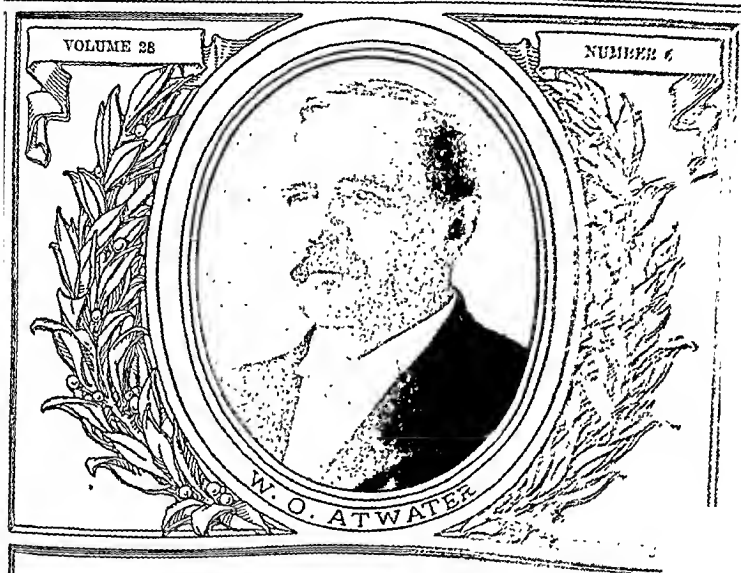
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# A STUDY OF PLASMA ASCORBIC ACID VALUES WITH RELATION TO THE TYPE OF DIET USED IN PUERTO RICO BY GROUPS OF INDIVIDUALS OF WIDELY VARIED ECONOMIC STATUS

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(Received for publication May 15, 1944)

The early work of Ashford ('29) in Puerto Rico and of Rhoads and his associates (Castle et al., '35), as well as of other investigators working elsewhere, has indicated that the sprue syndrome probably has its origin in a dietary imbalance of some sort. In connection with investigations being carried on at the University Hospital with patients exhibiting the sprue syndrome, plans were made to include studies on nutritional status with regard to the vitamins. In order to judge the significance of these findings it was necessary to make companion studies with representative samples of the population not suffering from sprue.

As encountered in Puerto Rico, sprue is found in individuals at all economic levels of living. The majority of cases come, naturally, from the low-income groups and, more especially, from that group having less than enough for satisfactory subsistence since it is the most numerous. The companion studies on non-sprue subjects were therefore planned to include groups of individuals giving sample populations under the two categories: (1) satisfactory income and (2) low income or no income.

During the course of the preliminary work, it became apparent that information on dietary habits was essential to an appraisal of the results to be obtained and a plan was accordingly made to include a record of dietary habits as a part of the study. The investigation as a whole included determinations of plasma carotene, vitamin A and ascorbic acid concentrations and urinary excretion of thiamine, riboflavin and factor F<sub>2</sub> (Holt and Najjar, '42). The present report covers the values obtained for plasma ascorbic acid concentrations of non-sprue subjects and shows how these are correlated with the data on dietary habits.



## METHODS AND PROCEDURES

*Determination of plasma ascorbic acid concentration*

Plasma ascorbic acid concentration was determined essentially according to the macro method of Farmer and Abt ('34-'35, '36) except for modifications in volume relations for extracting (Kastlin et al., '40).

Five to six milliliters of venous blood were drawn with a clean dry syringe and transferred immediately to a 15 ml. conical centrifuge tube containing 1 drop of a 20% potassium oxalate solution. After centrifugation, 2 ml. of the clear plasma were transferred to a second tube; 2 ml. of water and 4 ml. of 5% metaphosphoric acid solution were added and the tube was centrifuged. Two milliliter portions of the clear centrifugate were then titrated visually against a dilute solution of 2,6 dichloro-phenol-indophenol, care being taken to reach the end point within 5 to 10 seconds. Blank determinations for the metaphosphoric acid solution were always run parallel to the plasma determinations. The dye solution was standardized each day against a standard solution of pure ascorbic acid.

At the University Hospital and its clinics the blood samples were taken with the subjects in the postabsorptive state. Other subjects, who had eaten breakfast before the blood sample was taken, were instructed to omit citrus fruits or other foods containing vitamin C. In most cases the samples were analyzed immediately. When this could not be done they were stored in the refrigerator as the plasma-water-metaphosphoric acid mixture according to the suggestion of Kastlin et al. ('40) and Golden and Garfinkel ('42).

Blood samples from many subjects were taken at clinics some distance from the School of Tropical Medicine. Golden and Garfinkel ('42) reported that plasma in contact with blood cells might be kept at room temperature for as long as 3 hours with only slight loss of vitamin C. Our samples were never kept in excess of this time and the analyses were usually started within considerably less than 3 hours from the time the blood was drawn.

*Selection of subjects*

*Subjects having satisfactory income.* The first group under this category was made up of members of the staff of the School of Tropical Medicine and the University Hospital. As most of those selected receive a better than average salary for Puerto Rico, they have been treated together as one group rather than divided on an exact income basis.

The second group was made up of members of the staff and students in nutrition of the Department of Home Economics at the University of Puerto Rico in Rio Piedras. These subjects are of interest as reflecting the effect of nutrition teaching.

A third group, representative of the average citizen having sufficient income to buy food for an adequate diet, consisted of private patients in the University Hospital who came in for checkups or the treatment of minor ailments, and volunteers.

*Subjects having a low income or no income.* Four groups are included under this division. Two, groups 4 and 5, comprised individuals living in two housing projects of the Puerto Rico Rehabilitation Administration located relatively near San Juan. At the first project, known as St. Jnst, each householder has sufficient and suitable land for growing food crops; at the second, called Rio Plantation, there is land with each house but it is very poor and not suitable for crops of any kind except a few stunted coconut trees. The condition of the people at this project is most miserable indeed.

A third group, group 6, included individuals selected at one of the public health centers in San Juan.

There is a well-directed outpatient department at the University Hospital and the subjects in group 7 were selected from the patients attending the clinics there. Many of these individuals were sprue cases that had recovered and had received a certain amount of instruction in regard to diet.

### *Dietary histories*

With the members of the staff of the School of Tropical Medicine and the University Hospital, the staff and students of the Department of Home Economics at the University, and volunteers, the following plan was used for obtaining the dietary history: on the day before the blood sample was to be drawn, each individual was given a mimeographed form for recording (1) the foods eaten that day, (2) the foods eaten at breakfast the day of the examination, and (3) the types of fruits, vegetables, salads and other foods included in the diet regularly. In all other cases the personal interview method was used. For the groups at the two housing projects and the one group in San Juan the records were taken by the nurse in charge. From the subjects in the University Hospital and its clinics a complete history was taken covering not only food habits but all aspects of living that might have any significance in the interpretation of results.

## RESULTS

*Plasma ascorbic acid concentration*

A review of the literature indicated that most investigators consider a plasma ascorbic acid concentration in the region of 0.8 mg. per cent as compatible with a state of good nutrition but not necessarily of saturation. Renal threshold values, indicative of saturation, begin at slightly above 1.0 mg. per cent. The range for deficiency is somewhat arbitrarily given as beginning at 0.5 to 0.4 mg. per cent, while it is admitted by some that a person may have a zero plasma ascorbic acid concentration for a long period without clinical signs of scurvy. Clear-cut symptoms indicative of a chronic deficiency of the vitamin are still to be described, although reference is frequently made to sore and bleeding gums in this connection.

The values obtained for the plasma ascorbic acid concentration of the individuals in the seven groups were summarized according to ranges selected on the basis of these considerations and are presented in table 1. The range 0.50-0.79 mg. per cent was used to avoid a too sharp demarcation between the level generally taken as indicative of deficiency and that accepted as implying good nutrition. The use of a single figure for the transition point might have led to an incorrect classification of subjects with values close to this figure. In such cases more than one determination would be required to give the exact classification, a procedure outside the scope of this investigation.

In presenting table 1 attention is called especially to the similarity in the distribution of values for group 3 in the satisfactory income category and group 7 in the low income category. The first group, representing individuals with sufficient money to buy what they needed for good nutrition, gave nearly as many values in the low range as the second group having insufficient income for adequate food or any other essential commodity.

*Dietary habits*

The detailed data on dietary habits were analyzed on the basis of types of foods listed as common items in the diet. There were certain pronounced differences in the pattern diets for the seven groups that are of interest.

*(1) Foods other than fruits and salads*

For those in the satisfactory income category rice and beans was a common item in the diet except for the individuals of group 2. Vege-

TABLE 1

*Distribution of values for plasma ascorbic acid concentration determined on groups of individuals in Puerto Rico, of widely different economic status.*

PLASMA ASCORBIC ACID CONCEN- TRATION RANGE	INCOME SATISFACTORY			INCOME UNSATISFACTORY						PERCENT OF TOTAL
	Number cases			Number cases						
	Members staff Sch. Trop. Med. and Univ. Hosp. Group 1	Members staff students Dept. Home Ec. Univ. P.R. Group 2	Private patients Univ. Hosp. and volun- teers Group 3	Percent of total	Members of families in P.R.R.A. Housing Projects			Individuals selected by Social Service Worker San Juan Group 6	Patients Outpatient Depart- ment Univ. Hosp. Group 7	
					St. Just <sup>1</sup> Group 4	Rio Plantation <sup>2</sup> Group 5				
(milligrams percent)	(adults)	(adults)	(adults)	(adults)	(adults)	(adults)	(adults)	(adults)	(adults)	(adults)
(0.0-0.19)	(1)		(10)	(6.8)			(16)	(34)		(30.7)
0.0-0.49	11	6	20	26.7	11	34	18	14	36	30
0.5-0.79	15	14	12	25.4	2	3	5			12
0.8-0.99	10	13	5	47.8						6
1.0	17	19	7							7
Total	59	52	50		50	50	50	50		55

<sup>1</sup> Land suitable for food crops.

<sup>2</sup> Land not suitable for food crops.

tables of the starchy variety such as plantain and yautia were used frequently by all. Other types were listed frequently but not by all. Individuals in groups 2 and 3 had plenty of meat, milk, eggs, butter and cheese. These items were listed less frequently by group 1.

For the four groups in the unsatisfactory income category conditions relating to diet were varied. At the St. Just project, group 4, five of the children ate at the nursery and received rice and beans, starchy vegetables, and crackers often; cheese, butter, tomatoes and string beans once in a while. The other children ate at home with their families, and ate a diet which included chiefly rice and beans, starchy vegetables, codfish, and coffee with milk. Meat was eaten not more than once a week. At the Rio Plantation, group 5, all of the children received food at the school lunch room. The menu included evaporated milk, bread, rice and beans, codfish or canned beef once a week, eggs once a week, a starchy vegetable daily and some other vegetable, frequently canned, served as salad. The diet of the adults eating at home was similar to that for group 4 except that they had less food, the coffee was taken black, and meat was almost never eaten. The diet for group 6, individuals selected by a social service worker in San Juan, was similar to that of group 5 while the members of group 7 had a somewhat better diet, more like that of group 4.

## *(2) Fruits and salads*

The data for intake of fruits and salads were definitely related to the plasma ascorbic acid values and were summarized on that basis.

For the range 0.00-0.49 mg. per cent: Individuals of the three satisfactory income groups as a whole ate very little fruit. The children in groups 4 and 5, who ate at the nursery or the school lunch room received fruit or fruit juice once in a while. The other individuals in the four unsatisfactory income groups apparently ate almost no fruit.

For the range 0.50-0.79 mg. per cent: The use of fruit was reported occasionally by the members of groups 1 and 2 and frequently by group 3. Fruit juices were listed by members of group 3 as used frequently but were usually canned pear juice or canned peach juice. The children in groups 4 and 5 received fruit or fruit juice occasionally as mentioned above. The adults in group 4 of this range reported the use of salads. The one adult in group 5 was the janitress at the Public Health unit who included in her diet regularly a vegetable other than a starchy one or tomato soup. Individuals of group 7 reported the occasional use of fruit, chiefly oranges, grapefruits or bananas according to the price.

For the range 0.80-0.99 and above: Practically all individuals having a plasma ascorbic acid concentration greater than 0.80 mg. per cent reported the regular use of citrus fruits while they were in season and the frequent use of salads.

#### DISCUSSION

##### *Plasma ascorbic acid concentration*

In this investigation plasma ascorbic acid values were determined for 366 subjects. Of these, 161 comprised a group with sufficient income to enable them to have an adequate diet. Of the remaining 205, some had sufficient income for no more than a subsistence diet while the majority had little or no income. Of the 161 subjects, 43 or 26.7% had plasma ascorbic acid values within the range 0.00-0.49 mg. per cent and of the 205 remaining subjects, 169 or 82.4% came within this range.

All of the subjects studied were following their usual mode of living and there is no reason to believe that additional determinations, previous to or subsequent to the one made, would have given values differing from it by a significant margin. With the exception of the two groups made up of the members of the staff of the School of Tropical Medicine and University Hospital and the members of the staff and students of the Department of Home Economics at the University, all subjects were either patients under a doctor's care or who had been seen by a public health nurse or doctor at a clinic. As far as is known, none of them had been diagnosed as having symptoms of scurvy. Certainly none of the subjects in the two groups noted above, as exceptions, had any of the symptoms usually associated with scurvy and only one individual complained of bleeding gums.

A check on the question of the presence of subclinical symptoms of scurvy was obtained through the examination, by one of the authors (S), of all members of one group for signs of nutritional deficiency. These individuals, group 5, had been judged to be in the poorest condition nutritionally of all of those studied. Although many of these subjects had signs characteristic of undernutrition, none showed signs of perifollicular hemorrhages. There were no cases of ecchymosis or purpura and only occasional signs of gingivitis. In other words, there were no symptoms of scurvy or of severe vitamin C deficiency unless we accept as a symptom pyorrhea and loss of teeth from a poor condition of the gums. These were of frequent occurrence. There were several individuals with almost perfect sets of teeth and several with only one or two teeth missing from sets that showed practically no decay thus presenting contradictory evidence.

In the presentation of the results obtained for plasma ascorbic acid concentration a brief discussion is given of the more or less generally accepted concept of the state of vitamin C nutrition in relation to this factor. In their excellent paper on chemical methods for determining vitamin C deficiency Kastlin et al. ('40) go so far as to describe a plasma ascorbic concentration of 0.70-0.4 mg. per cent as indicating a mild deficiency of vitamin C and anything less than 0.40 mg. as associated with a state of severe deficiency.

In view of the evidence obtained with subjects in Puerto Rico there seems to be justification for asking what is meant by a mild deficiency of vitamin C? A severe deficiency of vitamin C? The further question is also raised: Are present methods for determining vitamin C require-

TABLE 2

*The effect of administration of ascorbic acid on the plasma ascorbic acid concentration. The administrations began after the initial value of 0.10 mg. % had been obtained on March 2nd.*

DATE	PLASMA ASCORBIC ACID CONCENTRATION	ASCORBIC ACID SUPPLEMENT GIVEN DAILY	
		From — To	Amount
	mg. %	dates	mg./day
March 2	0.10	March 2-10	50
March 11	0.31	March 11-24	50
March 25	0.56	March 25-April 21 (except 9 days)	50
April 22	0.44	April 22-May 5	50
May 6	0.53	May 6-20	50
		May 23-26	50
May 27	0.43	May 28-June 9	100
June 10	0.98	June 10-23	100
June 24	1.37	June 24-July 21	00
July 22	0.27		

ment either sound or adequate? It would seem that answers to these questions can be had only through more complete and extensive studies, than have yet been made, of subjects having plasma ascorbic acid values in what is now commonly described as the deficiency or near-deficiency range.

Further support for the justification of these questions was given by additional data collected during the course of the present study from one of the subjects, a clerk at the School of Tropical Medicine. On the first examination this man showed a plasma ascorbic acid concentration of 0.10 mg. per cent. Following the examination he was given a daily supplement of ascorbic acid over an extended period and plasma ascorbic acid determinations were made periodically with the results shown in table 2.

While receiving the supplements his plasma ascorbic acid concentration gradually increased to 1.37 mg. per cent but during the month following discontinuance of the supplement the concentration fell to 0.27 mg. per cent. This man, although small and underweight, is vigorous and healthy. His teeth are in good condition and there has been only one extraction and a few minor fillings. The mainstay of his diet is rice and beans and he eats little if any fruit.

Animals for experimental work are maintained in an adequately equipped animal house close by the School of Tropical Medicine. Under the conditions at hand blood samples for vitamin C analysis could be readily obtained from several species of animals and it seemed of interest to obtain these data in the belief that they might have a possible bearing on the question of plasma ascorbic acid concentration as an index of vitamin C deficiency. The values are given in table 3.

TABLE 3

*Plasma ascorbic acid values for different mammalian species.*

ANIMAL	PLASMA ASCORBIC ACID CONCENTRATION
	mg %
Dog	0.25
Goat	0.46
Guinea pig	0.56
Horse	0.46
Monkey	0.41
Rabbit	0.41

Without exception all values fall within the deficiency or near deficiency range described for man. All of the animals from which blood samples were taken were receiving what was believed to be an adequate diet and were healthy in all obvious respects. Unless we wish to assume that the plasma ascorbic acid concentration indicative of good nutrition is higher for man than for other species of animals these results seem also to justify raising the question of what, if any, level of plasma ascorbic concentration may be taken as indicative of vitamin C deficiency.

### *Dietary habits*

In Puerto Rico the dietary pattern is fairly uniform for the entire population; any modification is a result of insufficient income. For the well-to-do a breakfast consists of juice, cereal, eggs, bread or crackers, and coffee with milk. The main meal of the day includes soup, salad, meat, vegetables, rice and beans, and possibly a "dulce" or other des-



sert. Rice and beans are served by the well-to-do, even though they may not be eaten, and are simply returned to the kitchen to be consumed by the servants.

Among the people, as a whole, there is a preference for canned fruit juices such as apple, pear, peach, and prune juices, and apricot nectar, none of which are produced in Puerto Rico. Pears and apples are also considered among the choice fruits. This seems a little paradoxical in view of the fact that oranges, grapefruits, and pineapples are among the important crops of the Island. Many other fruits, rich in vitamin C, grow in Puerto Rico and might be grown in abundance. Salad vegetables, such as lettuce, tomatoes, peppers, cucumbers, and watercress are also major crops. In spite of this, canned asparagus, canned beets, canned peas, and canned green string beans are all too often used as the prime ingredients in salads.

When money for food is not adequate, fruit and salads are among the first items omitted from the diet. Breakfast is reduced to bread or crackers and coffee with milk. In the main meal, soup may still persist and may include most of the vegetables in the menu, except the starchy ones. Served in this manner, the vegetables have lost whatever vitamin C they may have had. The starchy vegetables are usually peeled and boiled, which means that they have lost much of their nutritive values. Rice and beans become a dish of major importance; meat is often replaced with salt codfish.

In the last extremity, the breakfast is reduced to crackers and coffee, which is usually black. There is only one other meal in the day, that eaten late in the afternoon. The items from which this is selected are rice and beans or rice alone, a boiled starchy vegetable or plaintain, and salt codfish.

From this brief survey it should be readily apparent that vitamin C is among the first of the nutrients to be reduced in amount in the diet of the Puerto Rican when money for food becomes inadequate.

#### SUMMARY

Plasma ascorbic acid determinations have been made for a total of 366 subjects, all citizens or long-time residents of Puerto Rico. These subjects were selected on the basis of seven groups of not less than fifty individuals each according to the income categories: (1) satisfactory income and (2) low income or no income. Information on food eaten the day preceding the examination and on dietary habits, in general, was also obtained.

The data from each of the seven groups were summarized on the basis of ranges for plasma ascorbic acid values usually accepted as indicating (1) severe deficiency of vitamin C, (2) near deficiency, (3) satisfactory nutrition, and (4) good nutrition.

Of the 366 subjects examined, 212 or 57.9%, had plasma ascorbic acid concentration values in the severe deficiency range. The high percentage of values in this range from one of the satisfactory income groups is emphasized as indicating that low income is not the only cause of vitamin C undernutrition.

The information on dietary habits reveals a close correlation between plasma ascorbic acid concentration and the presence of fruits and other vitamin C-rich foods in the diet.

The relation of level of plasma ascorbic acid concentration to deficiency of vitamin C and the requirement of this vitamin is discussed.

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# DIETARY ANEMIA IN THE PIGEON

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## FOUR FIGURES

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Dietary anemia in the pigeon has been studied previously by several investigators. Barlow ('27, '30) observed an anemia in pigeons fed polished rice; he attributed this to the result of inanition, which complicated the vitamin B<sub>1</sub> deficiency. Dameshek and Myerson ('40) used a ration of polished rice supplemented with 50 µg. of thiamine per bird daily. The anemia that developed in these pigeons responded to the administration of yeast concentrate or relatively crude liver extract, but a highly purified liver extract gave only partial cures. Extensive studies of pigeon anemia have been made by Hogan, Richardson, Johnson and Nisbet ('40), and Lee and Hogan ('42), using a purified basal ration deficient in the vitamin B-complex but supplemented with crystalline thiamine. These investigators reported that for complete recovery of both hemoglobin levels and body weight three different crude fractions are required: a fuller's earth adsorbate of rice bran extract, a fuller's earth adsorbate from crude liver extract, and the liver filtrate fraction.

The present study was undertaken with the object of obtaining further information on the nature of the unknown factors required for hemoglobin formation in the pigeon.

## METHODS

Pigeons bred for racing were used although some birds of mixed breed were included. They were housed in individual wire cages fitted with one-half inch mesh screen.

In a preliminary experiment, a group of 10 pigeons was fed polished rice, supplemented with salt mixture (U. S. P. no. 1) 0.1 gm. per day, Labco casein 1 gm. per day, cod liver oil 0.1 ml. per day, and cottonseed oil 0.3 ml. per day. The supplements were administered in gelatin capsules. In all subsequent experiments a purified diet similar to that

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of Hogan and co-workers was used. The diet fed had the following percentage composition: casein (Labco) 20, sucrose 73, salt mixture (U.S.P. no. 1) 4, and cottonseed oil 3. U.S.P. cod liver oil was given once weekly by capsule, 0.8 ml. per bird. The basal diet and water were kept before the pigeons at all times; no grit or other material was given.

Blood samples were taken at weekly intervals from one of the small peripheral wing veins. Acid hematin solutions were prepared for estimation of hemoglobin by the method of Schultze and Elvehjem ('34). The colorimetric readings were made in a Klett-Summerson photo-electric colorimeter (filter no. 42) which had been standardized with a series of blood samples of which the hemoglobin contents were obtained by the oxygen capacity method of Lundsgaard and Möller ('22). In the earlier studies weekly erythrocyte counts were also made with Hayem's fluid as diluent.

## RESULTS

### *Normal levels of hemoglobin and erythrocytes in pigeon blood*

In the majority of the pigeons the original hemoglobin values ranged from 14 to 18 gm. per 100 ml. The erythrocyte levels for 30 pigeons were from 2.98 to 4.16 million per cu. mm., with an average value of 3.65 million.

### *Preliminary experiments with a polished rice ration*

A group of 10 pigeons, 1 to 9 years in age, were fed polished rice supplemented with casein, salt mixture, cottonseed oil, and cod liver oil as described above. In accordance with the technique of Hogan et al. ('40), none of the vitamins of the B-complex were given for the first 30 days of the experimental period; during this time the weights of the pigeons decreased 26 to 36%. At this point 5 birds were fed 50 µg. of thiamine per day, whereas 5 received a daily supplement of 50 µg. thiamine, 60 µg. riboflavin, and 30 µg. pyridoxine. In 8 of the 10 birds the hemoglobin and erythrocyte levels decreased during the first 40 days. The average fall in hemoglobin was 81%, and in erythrocytes 85%. However, very surprisingly, within 1 or 2 weeks after the administration of thiamine was begun, either alone or in combination with riboflavin and pyridoxine, hemoglobin and red cells began to rise rapidly toward normal levels. Most of the weight lost was also regained, although at a somewhat slower rate. In none of the pigeons was there

any further fall in hemoglobin. Although the experimental period was extended to 167 days, the blood levels remained normal and the body weights were maintained well throughout the period of study.

Since anemia was not maintained under these conditions, probably because of the polished rice in the ration, the diet was modified accordingly.

### *Production of anemia with a purified diet*

*Experiment no. 1.* The use of the purified diet described above, proved to be successful in inducing anemia. Twenty pigeons were fed this ration; 5 were given 50  $\mu$ g. of thiamine each per day. The remaining pigeons were given 50  $\mu$ g. thiamine, 60  $\mu$ g. riboflavin, and 30  $\mu$ g. pyridoxine per bird per day. The majority of the surviving birds developed a significant anemia (arbitrarily chosen as 11 gm. or less, hemoglobin per 100 ml. of blood) within 126 to 140 days. The hemoglobin values at the low point of the anemia, before treatment, were from 6.5 to 10 gm. per 100 ml., with an average value of 7.9 gm. per 100 ml.

*Experiment no. 2.* In an effort to obtain more rapid development of anemia in a group of 19 pigeons (birds nos. 31 to 49), the purified diet described above was supplemented with 50  $\mu$ g. of thiamine daily after a depletion period of 4 weeks. This technique proved very satisfactory. Of the 16 birds which survived for an extended period 14 developed anemia (10 gm. hemoglobin or less per 100 ml.) within 60 to 123 days (with an average of 90 days); thus 74% of the pigeons placed on the experimental regime became anemic.

When a level of 8 to 10 gm. of hemoglobin per 100 ml. was reached, the bird was treated with various preparations to determine the effect on the hemoglobin concentration. In some cases riboflavin and pyridoxine were administered in addition to thiamine for a period of 2 to 4 weeks before the administration of test preparations. This addition of riboflavin and pyridoxine did not cause an increase in the concentration of hemoglobin.

The development of the anemia and effect of treatment is shown for a representative group of pigeons in figure 1. A dose of rice bran concentrate<sup>2</sup> of 1.0 ml. per day was found to cause a rapid increase of hemoglobin (pigeon 43), while doses as low as 0.25 ml. gave responses in some birds (pigeon 36). A rice bran eluate, prepared by the method

<sup>2</sup> Vitab Rice Bran Concentrate, obtained from the National Oil Products Company, Harrison, N. J.

of Lepkovsky, Jukes and Krause ('36), was tested on a number of birds. One ml. of the eluate represented 2 ml. of original rice bran concentrate. This material was effective in inducing hemoglobin production, as indicated by pigeons 43 and 47, figure 1. However, the amounts required were considerably larger than for the original extract. It was found that the elevation in hemoglobin levels produced

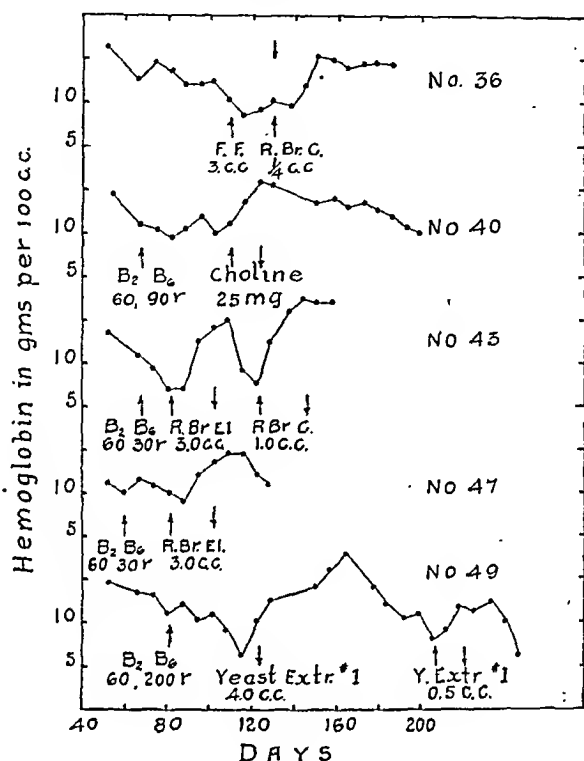


Fig. 1 Hemoglobin values for 5 pigeons in experiment no. 2. All birds received 50  $\mu$ g. of thiamine per day. Four of the birds received daily supplements of riboflavin and pyridoxine from the time indicated by the symbols  $B_2$  (riboflavin) and  $B_6$  (pyridoxine), the numbers below indicating the daily dose. The therapeutic materials administered were rice bran concentrate (R. Br. C.), rice bran eluate (R. Br. El.), choline, filtrate factor (F. F.) and yeast extract no. 1. The upright arrows ( $\uparrow$ ) indicate the points at which the administration of the various supplements was begun, while the immediately following inverted arrows ( $\downarrow$ ) indicate the withdrawal of the supplement. The figures below the symbols indicate the daily dose.

by 2 weeks of treatment was proportional to the dose over a considerable range. The average increases in hemoglobin in 2 weeks produced by daily doses of 0.375, 0.75, 1.5 and 3.0 ml. of the rice bran eluate were 0.0, 1.9, 2.4 and 4.3 gm. of hemoglobin per 100 ml. of blood, respectively. Two pigeons were used for the 1.5 ml. dose, 3 to 5 for the other levels.

Preliminary experiments with a yeast extract were also made on these pigeons. Dried yeast<sup>3</sup> was extracted with 25% alcohol and the extract was concentrated in vacuo. The final volume was adjusted so that 1 ml. represented 1 gm. of yeast (Yeast Extract no. 1). The comparative effects of small and large doses of this preparation are shown in the curve for pigeon 49, figure 1. It is seen that 4.0 ml. given daily for 1 week produced a very large hemoglobin response, an increase of 12 gm. of hemoglobin per 100 ml. of blood being attained in 6 weeks. At a later date a daily dose of 0.5 ml. of the same extract was given to this pigeon for 2 weeks and this elicited a slightly lower hemoglobin increase (3.7 gm.). Further experiments with other pigeons confirmed the activity of this yeast extract in stimulating hematopoiesis (see experiment 3).

To determine the activity of other vitamin B-complex factors a number of the birds, after 100 to 160 days on the experimental ration, were given a daily supplement containing thiamine 50 µg., riboflavin 100 µg., pyridoxine 50 µg., pantothenic acid 200 µg., and nicotinic acid (niacin) 1000 µg. per pigeon. In most birds this dietary supplement produced little or no effect on hemoglobin concentrations and demonstrated that the anemia was not primarily due to a lack of any of these five synthetic factors.

*Experiment no. 3.* In order to keep the birds in good general condition and to produce a more specific deficiency of the anti-anemia substance, the following plan was used: A group of 36 pigeons was fed the basal vitamin B-complex-free purified diet given above, with no addition of any of the B factors for the first 3 weeks. At this point, supplementation was begun with 50 µg. of thiamine per pigeon per day. After an additional 4 weeks, riboflavin, pyridoxine, calcium pantothenate, and nicotinic acid, in the amounts given in the preceding paragraph, were fed in addition to the thiamine. This supplement was then continued unchanged for the remainder of the experiment.

The 36 pigeons placed on the experiment had an initial hemoglobin level of 15 gm. per 100 ml. of blood, or higher. There was a rapid fall from this level during the first 7 weeks while the birds received none of the B factors other than thiamine. After 7 weeks, the hemoglobin had fallen to levels of 12.0 gm. per 100 ml. or lower with a range of 8.5 to 12.0 gm. in 23 of 34 surviving birds.

In most of the pigeons there was a cure of anemia lasting for several weeks after the administration of the complex supplement containing

<sup>3</sup> Pure dehydrated yeast, Northwestern Yeast Company, Chicago, Ill.



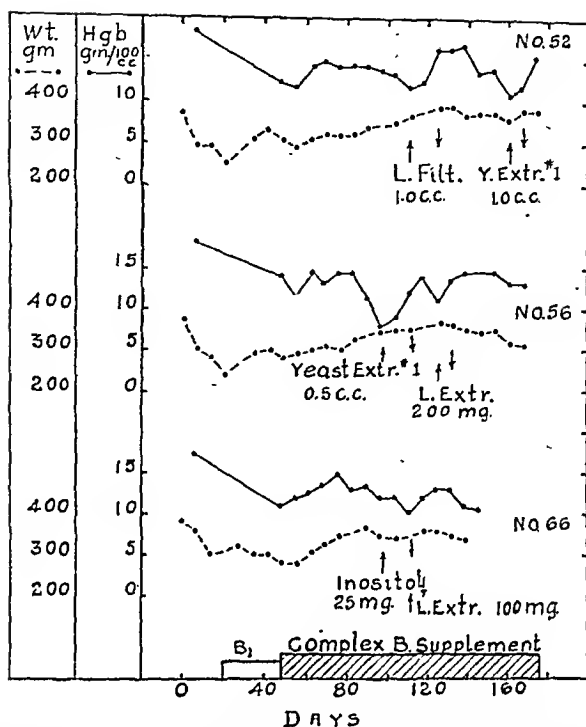


Fig. 3 Hemoglobin and body weight levels in experiment no. 3. The vitamin B-complex supplement was the same as in figure 2. The 1.0 ml. dose of liver filtrate contained 400 mg. of solids.

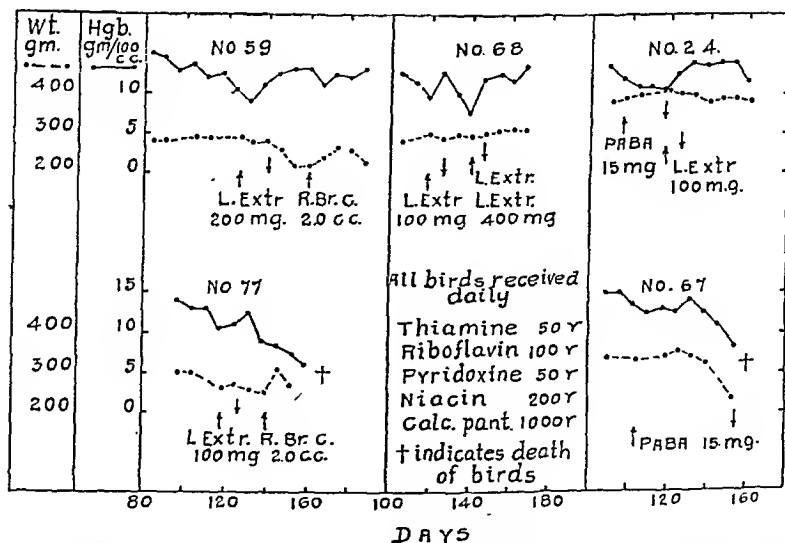


Fig. 4 Response of hemoglobin and body weight levels of anemic pigeons to treatment with liver extract, rice bran concentrate (R. Br. C.) and p-aminobenzoic acid (P A B A).

TABLE 1

*Hemoglobin regeneration with liver filtrate preparation.**All pigeons were given a daily dose of 1.0 ml., containing 400 mg. of solids.*

PIGEON NO.	DURATION OF TREATMENT	HEMOGLOBIN LEVEL AT START OF TREATMENT	HEMOGLOBIN LEVELS AFTER 1 TO 3 WEEKS TREATMENT			INCREASE IN HEMOGLOBIN LEVELS PRODUCED IN		
			One week	Two weeks	Three weeks	One week	Two weeks	Three weeks
	Weeks	gm./100 ml.		gm./100 ml.			gm./100 ml.	
52	2	11.8	12.3	16.3	16.5	0.5	4.5	4.7
60	3	7.9	11.8	7.2	7.5	3.9	-0.7	-0.4
69	3	10.0	7.1	7.9	15.0	-2.9	-2.1	5.0
72	3	10.3	11.4	11.9	14.8	1.1	1.6	4.5
76	2	12.0	12.7	15.4	13.5	0.7	3.4	1.5
99	3	11.3	10.8	12.6	12.7	-0.5	1.3	1.4
102	2	10.4	14.2	14.0	12.6	3.8	3.6	2.2

*Body weight changes*

In general, there was little correlation between body weight levels and the state of anemia. Following the period of increased hemoglobin production induced by changing the basal vitamin supplement from thiamine alone to the five synthetic factors, in most birds the hemoglobin levels began to fall fairly rapidly, starting usually some time from the eightieth to the hundredth day. During this period of declining hemoglobin levels the body weight either increased (birds 52 and 56, fig. 3), or remained approximately constant, as in pigeons 61 and 70 (fig. 2) or pigeons 59 and 68 (fig. 4).

The treatment of anemic pigeons with daily doses of 100 to 200 mg. of liver concentrate powder, although effective in raising hemoglobin levels, in most cases had little or no effect on the body weight. This is shown well in the curves for pigeons 24, 59, 68 and 77 (fig. 4). In contrast to this, a 2.0 ml. daily dose of rice bran concentrate in every case produced a 20 to 50 gm. increase in weight during a 2-week period, even though this amount of material supported increased hemoglobin levels in only part of the birds. This is shown in figure 2, pigeons 61 and 70 and in figure 4, birds 59 and 77. These results, along with similar findings on other birds, suggest that there are at least two deficiencies in the basal ration supplemented with thiamine, riboflavin, pyridoxine, nicotinic acid, and pantothenic acid; that is, an anti-anemic factor and a weight-promoting factor.

*Symptoms of neuromuscular dysfunction*

In a number of birds after a period of 150 days or longer on the deficient ration a state of incoordination developed. The affected

birds were ataxic, i.e., tottered when trying to walk and were unable to fly. Muscular tone was normal, since this condition appeared in birds that were plump as well as those that were emaciated. Occasionally one of the affected birds was observed to have a convulsive attack lasting for about a minute. Following the convulsive episode, the muscles were relaxed. Large doses of thiamine were ineffective in preventing the convulsive seizures. Most of these birds were treated with large doses of yeast extract, rice bran concentrate, or liver extract as soon as the symptoms were observed. Despite this therapy the affected birds invariably died, usually within 2 to 4 days.

#### DISCUSSION

The prompt response of anemic pigeons fed a vitamin B-complex deficient purified diet to vitamin complex extract from yeast, liver, and rice bran demonstrates the existence of a nutritional anti-anemic factor for the pigeon. Several considerations support this view. One of these is the very rapid increase in hemoglobin levels as the result of yeast or liver extract therapy. An increase of 3 to 5 gm. of hemoglobin per 100 ml. in 1 week is usually obtained with an optimum dose of an active extract. The fall in the hemoglobin level after the withdrawal of the active supplement is frequently almost as rapid as the previous increase. In addition, it may be noted that the factor responsible for the increase in hemoglobin level has very little relation to maintenance of body weight in that frequently pigeons continue to gain in weight while the red blood cell and hemoglobin values are falling. Likewise, doses of liver extract sufficiently large to produce satisfactory hemopoietic stimulation as a rule induce little or no increase in body weight. The data suggest that pigeons require, in addition to the unknown anti-anemia factor, a weight restoration factor.

It has not been possible to identify the anti-anemic agent with any of the known members of the vitamin B-complex. In addition to the vitamins in the basal diet, p-aminobenzoic acid and inositol have been ruled out by therapeutic trial. No experiments were carried out with crystalline biotin or "folic acid."

One must consider the possible similarity of this anemia of pigeons with that described in chickens by Hogan and Parrott ('40) though there may be some differences in behavior of the active principle. In the present study yeast extracts have been very effective in promoting blood formation, whereas 4% of acid-hydrolyzed yeast was used in the anemia-producing diet for the chickens. A further point of difference is that the chick anti-anemic substance is adsorbable on fuller's earth

at pH 1, while it has been found that the pigeon factor in liver extract is very poorly adsorbed on fuller's earth at pH 4.0. It is possible, of course, that this dissimilarity in adsorption may to some extent be due to the difference in pH. Pfiffner and coworkers ('43) have recently reported that the chick anti-anemic factor is identical with Williams' "folic acid."

The possible relation between this anemia of pigeons and that of certain dietary anemias in mammals may also be considered. Anemia which is normocytic, or in a few cases macrocytic, has been produced in several species fed diets lacking the filtrate portion of the vitamin B-complex. Thus Fouts, Helmer and Lepkovsky ('40) found that 6 of 7 dogs lacking filtrate fraction developed a moderate anemia tending toward the macrocytic type. Chick et al. ('38) have reported a normocytic anemia in hogs deprived of filtrate factor. Likewise, Wills, Clutterbuck and Evans ('37) produced a macrocytic anemia in monkeys fed a deficient diet composed of natural foodstuffs and they found that the curative factor in yeast remained in the filtrate upon treatment with fuller's earth.

Thus, the similarity in adsorbability and the common distribution in yeast and liver extracts suggest that the anti-anemic agent in the filtrate fraction of the B-complex active in pigeon anemia may be the same as that reported active in chick and mammalian anemias.

#### SUMMARY

A severe anemia develops in pigeons maintained on a purified ration containing thiamine, riboflavin, pyridoxine, nicotinic acid, and calcium pantothenate, but lacking other members of the vitamin B-complex. The hemoglobin levels may be restored to normal by administering extracts of yeast, of liver, and of rice bran. The anti-anemic agent in a solution of liver extract remains in the filtrate upon treatment with fuller's earth at pH 4.0.

Evidence is also presented to demonstrate that a second or weight-restoration factor is required by the pigeon.

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The samples were washed, wiped with clean towels and cut into small pieces which were then mixed. Portions for the cooked and raw samples were weighed immediately, the sample for cooking being weighed directly into the dish in which it was cooked and dried. The Chinese custom of using fat in cooking was omitted to avoid extraction before analysis. The foods were cooked with a small amount of distilled water until they approximated the "doneness" at which they are ordinarily eaten. The green leaves require 5 to 10 minutes, turnip and white gourd about  $\frac{1}{2}$  hour. Soybeans were ground, as they are when used in soybean cereal flour mixtures in North China, sufficient 1% bicarbonate solution was added to make a paste, and the mixture then steamed over water. The practice of removing the tough outside layers of the stems of rape and kan lan ts'ai was followed. Cooking water was not discarded and all samples were dried under vacuum at 40°C., after which they were pulverized in a mortar. Since the other vegetables contain little fat, they were analyzed without extraction with ether, which was necessary with soybeans. In the analyses, the method of William and Olmsted ('35) was closely followed.

#### RESULTS AND DISCUSSION

Except for the dried seeds all of the foods studied had high moisture contents, ranging from 82 to 96%, with the majority above 90 (table 1). For this reason the total fiber of these foods represented only 0.4 to 1.8% of the wet weight, for both raw and cooked samples, although on the dry weight basis the values ranged from 8.5 to 19.2%. Significant amounts of complex carbohydrates were not found in rice, which evidently had been highly polished; the amounts in millet compare with those in the other foods on the wet basis, but are a much smaller fraction of the dry weight; soybeans had a much higher concentration on the wet basis and near the average of the amounts in the other foods on the dry basis.

The three samples of rape showed differences in total complex carbohydrate content which seem attributable principally to variations in the cellulose and water present. The two samples of mustard also showed differences in complex carbohydrate composition and water content. The differences between the fiber contents of samples of the same food are small in comparison with the variations found in other food components, owing to degree of hydration, age, storage conditions, species and soil conditions.

On the wet basis lignin was found in amounts ranging from less than six hundredths (0.06) to 0.5%, however, 1.4 to 3.2% of the dry ma-

TABLE 1

Complex carbohydrate, water, protein, fat and ash contents of Chinese foods. Per cent of dry and wet weights of raw and cooked samples.<sup>1</sup>

FOOD	MOIS- TURE	LIGNIN		CELLU- LOSE		Hemicellulose		TOTAL COMPLEX CARBOHYDRATE		PROTEIN %		FAT %		ASH			
		Dry	Wet	Dry	Wet	Total	Stable	Dry	Wet	Dry	Wet	Dry	Wet	Dry	Wet		
<b>Leafy vegetables</b>																	
Raw	91	2.2	0.2	7.7	0.7	4.0	0.1	2.8	0.2	14.6	1.3	37.3	3.4	3.2	0.3	17.4	1.6
Cooked	91	2.2	0.2	7.9	0.7	4.1	0.4	2.4	0.2	14.2	1.3	36.9	3.3	..	..	..	..
Raw	93	2.6	0.3	7.8	0.5	5.3	0.3	2.7	0.2	15.6	1.1	36.7	2.5	4.2	0.3	14.7	1.0
Cooked	92	2.4	0.3	6.1	0.3	4.6	0.3	2.3	0.2	13.1	1.0	35.6	2.7	2.7	0.2	12.2	0.9
Raw	93	2.6	0.2	7.8	0.3	5.0	0.4	2.7	0.2	12.4	0.9	34.7	2.5	4.0	0.3	13.2	1.1
Raw	94	1.6	0.1	6.1	0.1	2.6	0.1	2.2	0.1	12.1	0.7	..	..	..	..	..	..
Raw	94	1.8	0.1	6.9	0.1	4.0	0.2	2.5	0.2	12.7	0.7	25.0	1.1	..	..	..	..
Raw	96	1.4	0.1	9.4	0.4	1.9	0.2	3.1	0.2	15.8	0.7	19.4	0.8	2.1	0.1	13.1	0.6
Raw	89	2.6	0.3	6.9	0.7	5.3	0.5	4.0	0.4	14.7	1.6	36.5	1.9	3.3	0.3	17.9	1.8
Cooked	89	2.4	0.3	5.9	0.7	4.3	0.3	3.1	0.4	12.6	1.4	33.5	1.8	2.9	0.3	14.7	1.7
Raw	92	2.0	0.2	7.4	0.6	4.1	0.3	3.2	0.3	13.7	1.0	26.4	2.0	1.3	0.1	15.5	1.3
Raw	80	1.7	0.2	7.1	0.8	3.9	0.4	2.8	0.3	13.0	1.4	36.5	4.0	2.5	0.3	13.9	1.5
Cooked	88	1.4	0.2	7.8	0.8	3.3	0.4	2.4	0.3	12.5	1.4	36.3	4.2	1.0	0.3	13.6	1.6
Raw	92	2.0	0.2	6.9	0.6	5.3	0.4	2.6	0.2	14.8	1.2	35.0	2.8	2.4	0.2	18.0	1.5
Cooked	91	2.1	0.2	5.4	0.5	5.0	0.4	2.6	0.2	13.3	1.1	35.3	3.0	2.0	0.2	17.0	1.5
Raw	90	2.0	0.2	9.4	0.9	4.0	0.4	3.3	0.3	15.4	1.5	27.6	2.7	..	..	..	..
Cooked	92	2.2	0.2	9.7	0.8	3.9	0.3	3.1	0.3	15.4	1.3	..	..	..	..	..	..
Raw	87	3.2	0.4	6.5	0.8	4.3	0.6	2.8	0.4	13.9	1.6	..	..	..	..	..	..
<b>Other vegetables</b>																	
Raw	90	0.4	0.0	11.2	0.6	4.5	0.2	4.1	0.1	19.2	0.8	17.8	0.8	..	..	..	..
Cooked	90	0.3	0.0	13.6	0.6	3.8	0.2	3.1	0.1	17.0	0.8	..	..	..	..	..	..
Raw	95	1.0	0.0	4.5	0.2	1.5	0.2	2.1	0.1	10.0	0.4	..	..	..	..	..	..
Cooked	95	1.0	0.0	5.1	0.2	1.7	0.2	2.4	0.1	10.1	0.4	..	..	..	..	..	..
Raw	93	0.9	0.1	7.9	0.6	3.2	0.2	2.5	0.2	12.0	0.9	13.2	1.0	0.8	0.0	10.0	0.7
Cooked	93	0.4	0.0	7.9	0.5	2.7	0.2	1.7	0.1	11.0	0.8	13.9	1.0	2.2	0.2	10.2	0.8
Raw	94	1.5	0.1	7.8	0.4	5.2	0.4	3.9	0.2	14.5	0.8	41.1	2.1	..	..	..	..
Raw	94	1.1	0.1	6.9	0.4	3.4	0.2	2.7	0.2	11.7	0.6	39.1	2.2	1.5	0.1	4.1	0.2
Raw	82	1.6	0.3	4.8	0.4	3.1	0.4	1.7	0.3	8.5	1.5	10.3	1.8	0.6	0.1	5.0	0.9
Cooked	82	1.6	0.3	5.1	0.9	1.9	0.3	1.8	0.3	8.6	1.6	9.6	1.7	0.6	0.1	5.0	0.9
Raw	89	1.0	0.1	7.4	0.8	4.8	0.5	3.6	0.4	13.2	1.5	27.5	2.1	1.2	0.1	5.2	0.6
Cooked	89	1.0	0.1	7.0	0.8	3.8	0.4	2.3	0.2	11.8	1.3	26.1	2.0	1.0	0.1	5.2	0.8
<b>Pre-soaked seeds</b>																	
Raw	80	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	7.3	6.8	0.4	0.4	0.5	0.5
Raw	7.5	0.5	0.5	0.0	0.0	0.4	0.9	0.6	0.5	1.5	1.4	11.5	10.6	4.1	3.8	1.7	1.6
Raw	4.4	0.1	0.3	4.6	4.1	7.3	6.0	4.8	4.5	12.3	10.7	53.6	51.3	..	..	..	..
Cooked	4.0	0.1	0.3	4.3	4.1	7.0	5.7	3.6	3.5	8.6	8.2	52.2	50.1	..	..	..	..
Raw	4.0	0.4	0.3	1.9	1.8	2.3	2.2	2.3	2.3	2.8	2.8	54.6	52.6	..	..	..	..

<sup>1</sup> The values have been rounded to one decimal place. Zeros do not indicate an absence of complex carbohydrate, but merely amounts less than one-tenth per cent.<sup>2</sup> Nitrogen content times 6.25<sup>3</sup> Ether extracted.<sup>4</sup> Pooled sample (raw and cooked).



# VARIATION IN THE ASCORBIC ACID REQUIREMENTS FOR SATURATION OF NINE NORMAL YOUNG WOMEN<sup>1</sup>

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Although numerous publications have appeared, a compilation of published data shows that there remains a considerable variation in suggested requirements for the ascorbic acid saturation of normal adults. Much of the variation is due to the fact that several methods of measuring saturation requirements have been used. These methods have included a plasma ascorbic acid method, urinary responses to ascorbic acid in test doses, and "utilization" or "retention" values of the vitamin. Due to the fact that most of these methods are very time consuming, a rather small number of subjects has been studied.

Using the criterion of a urinary response to a test dose of ascorbic acid given after the subject has been maintained on a controlled diet at a definite level of ascorbic acid intake, Belser, Hauck, and Storvick<sup>1</sup> ('39) studied 7 adults, reporting their requirement for body saturation to be from 1.0 to 1.6 mg. per kg. of body weight; Todhunter and Robbins ('40) studied 3 adults by the same method, reporting their requirement for saturation to be 1.6 to 1.7 mg. per kg. per day. The period on a controlled diet followed a saturation period in which the subject was given massive doses of ascorbic acid to insure saturation of the body tissues. In these studies the lowest response to the 400-mg. test dose after known saturation was used as the criterion for saturation for a subject. Since most of these responses were 50% or more of the 400 mg. intake, most later studies use the 50% level as their standard, and it is taken as the criterion for saturation in this paper.

Using urinary responses to the test dose and also ascorbic acid levels in the blood, Storvick and Hauck ('42) reported data on 5 subjects. The amounts of ascorbic acid necessary for tissue saturation (using the 50% level of urinary excretion) for these subjects were 1.33, 1.32, 1.34, 2.95 and 1.51 mg. per kg. of body weight.

<sup>1</sup> From a dissertation presented to the Graduate School of the Virginia Polytechnic Institute by Alice B. Kline in partial fulfillment of the requirements for the M. S. degree.



Urine collections were made only during the 24-hour periods preceding and following the test dose. Since conclusions on saturation are drawn only from the urinary response to test doses, daily analyses of urine were omitted in order to permit the study of more subjects. Analyses were made by the same method used for the foods (Thornton, '38).

Each experimental period consisted of a 5-day saturation period followed by 6 days on the experimental level. During the saturation period 200 mg. of crystalline ascorbic acid<sup>2</sup> was given each subject for 4 days. On the fifth day a 400-mg. test dose was given and if 50% or more of the total intake for that day was excreted in 24 hours, the subject was considered saturated. On the fourth and fifth days of the saturation period the subjects ate the controlled diet containing approximately 16 mg. of ascorbic acid but for the first 3 days of the period the diet was not controlled. If the subject was saturated she was continued on the controlled diet and the experimental level of ascorbic acid was begun. This was continued for 6 days. On the seventh day the 400-mg. test dose was again given and if 50% of the 400 mg. appeared in the urine, it was considered that the level of the previous 6-day intake had been sufficient for the subject's saturation needs. All crystalline supplements were given at breakfast in water which was colored and flavored to resemble orange juice. Since a range of 1.0 to 1.95 mg. per kg. had been found required for most of the individuals studied, a level of 1.8 mg. per kg. was used for the first period in the present study. If the subject was saturated on this level, 1.4 mg. per kg. was given next. If a subject was not saturated at this level it was assumed that her requirement for saturation was between 1.4 and 1.8 mg. per kg. However, if a subject was not saturated at the 1.8 mg. level she was put on an intake of 2.2 mg. per kg. If a subject was saturated at the 1.4 mg. level she was continued on a lower level. Thus, the saturation requirement for any individual could be found by a continuation of the above process.

#### RESULTS AND DISCUSSION

All 9 subjects were put on the saturation level at the beginning of the experiment but all did not become saturated during this 5-day period. It was interesting to note that out of the 5 subjects who did not excrete as much as 50% of the test dose, 4 were individuals who had not been habitual in coming to breakfast previous to the study. Since the chief source of vitamin C for the day was usually served at breakfast, the

<sup>2</sup> Acknowledgment is made to Hoffman LaRoche, Inc. for a generous supply of crystalline ascorbic acid.

reason for the lack of saturation is obvious. It was, of course, necessary to continue the saturation level for these individuals until the tissues were saturated before experimental levels could be started.

The responses of the 9 subjects to various levels of intake are given in table 2. It may be seen that 7 of the subjects, all except I and VI, were

TABLE 2

*Twenty-four hour excretions of a test dose of 100 mg. of ascorbic acid (total intake = 100 + am't. in diet) following two saturation periods and periods of various levels of ascorbic acid intake.*

SUBJECT NO	SATURATION LEVEL				VARIOUS EXPERIMENTAL LEVELS							
	200 mg.		0.6 mg. per kg. body wt.		1.4 mg. per kg. body wt.		1.6 mg. per kg. body wt.		2.2 mg. per kg. body wt.			
	Period	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.	exc'd.
		mg.	%	mg	%	mg	%	mg	%	mg	%	
I	1	225	54					80	21	200	48	
	2	186	45									
II	1	251	60			165	40	303	73			
	2	357	86									
III	1	240	58			177	43	226	54			
	2	336	81									
IV	1	194	47			187	45	286	69			
	2	352	85									
V	1	294	71			178	43	206	49			
	2	246	59									
VI	1	230	55					165	40	140	34	
	2	135	33									
VII	1	254	61			200	48	331	80			
	2	223	54									
VIII	1	336	81	233	56	370	89	218	52			
	2	350	84									
	3	366	88									
IX	1	260	63			191	46	315	76			
	2	237	57									

saturated at the level of intake of 1.8 mg. per kg. When the level of intake was reduced to 1.4 mg. per kg., 6 of these individuals became unsaturated. Thus, for these 6 young women, an intake above 1.4 but not greater than 1.8 mg. per kg. was required for saturation.

Three of the nine subjects had either higher or lower requirements than 1.4 to 1.8 mg. per kg. Subject VIII excreted 89% of the test dose after a level of 1.4 mg. She was continued on a reduced level of 0.6 mg.

per kg. Her response to the test dose after this level was 56% of her intake. Thus, she was still maintaining tissue saturation on this low level. Time did not permit further study of this individual but certainly her requirement was far below that usually recommended. The reasons for such deviation in requirements for ascorbic acid saturation are at present unknown.

Subjects I and VI were not saturated at 1.8 mg. so they were put on a level of 2.2 mg. per kg. On the latter level, subject I excreted 48% of the test dose and subject VI, 34%. It was not possible to continue study of these 2 individuals but it would seem that their requirements for saturation had not yet been met.

Thus, from the present study we find that of the 9 young women, 6 had a requirement of ascorbic acid for saturation of 1.4 to 1.8 mg. per kg., 1 had a saturation requirement of 0.6 mg. or less, and 2 of 2.2 mg. or more. No report in the literature was found of a requirement for saturation as low as 0.6 mg. per kg. and the reasons for such a low requirement for this young woman are not known. Storvick and Hauck ('42) found that 1 of their 6 subjects had a requirement of 2.85 mg. per kg. which is interesting in view of the fact that 2 subjects in the present study were found to require 2.2 or more mg. per kg. for saturation. It is felt that by using the simplified technique proposed in this study, large numbers of individuals could be studied and the extent of variation in requirements for saturation could be learned.

#### SUMMARY

1. A simplified modification of the method using responses to the test dose for the determination of saturation needs of ascorbic acid was used. Daily urinalysis for ascorbic acid were omitted and only the 24-hour samples of urine before and after the test dose were analyzed. A more liberal intake of ad libitum foods was allowed and the subjects as far as possible lived and ate under their normal dormitory conditions.

2. Of 9 young women, 6 were found to have an ascorbic acid requirement for saturation above 1.4 mg. per kg., but not greater than 1.8 mg. One subject had a requirement of 0.6 mg. or less per kilogram, while 2 had requirements of 2.2 mg. or above per kilogram.

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# THE EFFECT OF SPRAY-DRYING AND THE SUBSEQUENT STORAGE OF THE DRIED PRODUCT ON THE VITAMIN A, D, AND RIBOFLAVIN CONTENT OF EGGS

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ONE FIGURE

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The recent and unprecedented use of dried egg in the human diet has made it imperative to evaluate accurately its nutritive properties. The effect of dehydration on the vitamin content of eggs is a major consideration because of the recognized value of eggs as a protective food.

Hauge and Zscheile ('42) found that the spray-drying process caused little or no loss of vitamin A in eggs. Klose, Jones and Fevold ('43) confirmed this finding, and, in addition, observed that thiamine, riboflavin, pantothenic acid, and nicotinic acid were stable during the spray-drying process. They also reported that during subsequent storage some loss of thiamine occurred and vitamin A was lost fairly rapidly, particularly at elevated temperatures.

A study of the effect of spray-drying on the vitamin A, D, and riboflavin content of eggs and the stability of these vitamins during subsequent storage of the dried product was undertaken at the Beltsville Research Center, Beltsville, Maryland, in May, 1942.

## MATERIALS AND METHODS

The samples of fresh liquid and dried eggs were obtained through the cooperation of a commercial egg drying plant in Kansas City. In order to insure proper sampling, samples of fresh liquid eggs were taken from the churn and a few minutes later samples of the dried product were obtained. The samples were frozen and shipped under refrigeration to Beltsville.

The vitamin A content of the eggs was estimated by biological assay with rats and by the spectrophotometric method, vitamin D by biological assay with rats, and riboflavin by the microbiological method.

The general procedure for the biological assay with rats was adapted from the method described in the U. S. Pharmacopoeia XII.

For the vitamin A estimations, rats between the ages of 21 and 26 days were collected in groups in screen-bottom cages and were housed in air-conditioned rooms. They were fed only the basal vitamin A deficient diet. After a depletion period of 3 weeks the animals were placed in individual screen-bottom cages. When the rats reached a constant weight, or by the twenty-eighth day, supplementary feeding of the test material was started and continued for a period of 6 weeks. Weekly weights were recorded for all the animals. Because the eggs used were received in three batches over a period of months, it was necessary to make three separate assays. Usually two to six samples of egg material were assayed together. When six samples were to be assayed, ten groups, each containing four male and four female rats were assembled and distributed isogenically according to weight. A sample of egg was fed to each of six of the groups and a reference standard fed at appropriate levels to the other four groups.

Crystalline  $\beta$ -carotene, dissolved in peanut oil and checked for purity and concentration by means of the spectrophotometer, was used as a standard of reference. The standard was fed daily at levels equivalent to 0.5, 1.0, 2.0, and 3.0 International Units of vitamin A. The results obtained from the growth of the groups of rats fed the standard were used to establish a curve of response. The vitamin A content of each egg sample was estimated from this curve.

The "line" test curative method was used for the estimation of vitamin D. The rats were from the same stock colony as those used for the vitamin A assays. The basal rachitogenic diet, no. 2965, of Steenbock and Black ('25), was fed during the depletion period of 21 to 28 days. The vitamin D-depleted rats were then distributed isogenically into groups, and received supplements of U.S.P. reference cod-liver oil and egg material for 6 to 7 days. The animals were killed and tested for the degree of bone healing on the tenth day after supplementary feeding was begun. A curve of response was established from the means of the bone healing values which resulted from feeding different levels of the reference oil. The vitamin D content of the egg samples was estimated from the response curve. (An arbitrary standard scale extending from 0 to 3 with graduations of 0.5 was used in the estimation of the degree of bone healing.)

The procedure used in the spectrophotometric estimation of vitamin A was essentially that described by Madsen and Davis ('38), except that

purified ethyl ether was used instead of petroleum ether to extract the non-saponifiable fraction.

For the microbiological estimation of riboflavin the egg samples were autoclaved in 0.1 N hydrochloric acid at 15 pounds pressure for 30 minutes. After adjustment of the pH to 4.5 with sodium hydroxide the solution was filtered and the filtrate made up to a suitable volume and assayed by the method of Snell and Strong ('39).

#### EXPERIMENTAL RESULTS

Dehydration did not cause an appreciable loss in the vitamin A content of emulsified whole egg. The results of both biological assays and physical determinations show good agreement between the fresh and dehydrated samples (table 1). However, the values obtained by the biological assay of egg yolk indicate that drying of this material caused some destruction of the vitamin A.

TABLE 1

*Effect of spray-drying on the vitamin A, D, and riboflavin content of eggs  
(all figures on a dry weight basis).*

DESCRIPTION OF SAMPLE		VITAMIN A		VITAMIN D	RIBOFLAVIN
		Biological	Physical	I. U. per gm.	μg per gm.
		I. U. per gm.	μg per gm.	I. U. per gm.	μg per gm.
No. 1	Emulsified whole egg	61	31	4.1	14
	Spray-dried whole egg	63	31	3.8	13
No. 2	Emulsified egg yolk	95	39	8.1	9
	Spray-dried egg yolk	64	37	8.6	9
No. 3	Emulsified whole egg	50	21	3.3	13
	Spray-dried whole egg	42	22	4.0	14

The experimental results indicate that drying caused little or no loss in the vitamin D and riboflavin content of the eggs. The variations between the values for the emulsified and dehydrated eggs are slight and may be attributed to experimental error.

The decrease in the vitamin A content of stored spray-dried eggs is shown in table 2. The results of both the biological and physical methods show a considerable loss, the results of the former method indicating a much greater loss than those of the latter. This difference is especially noticeable in the case of the samples stored at the higher temperatures.

Vitamin D and riboflavin in the stored spray-dried eggs were quite stable during storage periods of from 4 to 6 months at temperatures up to 98°F. (table 2).



TABLE 2

*Effect of storage on the vitamin A, D, and riboflavin content of spray-dried eggs  
(all figures on a dry weight basis).*

CONDITIONS OF STORAGE		VITAMIN A		VITAMIN D	RIBOFLAVIN
Time	Temperature	Biological	Physical		
Months	°F.	I. U. per gm.	I. U. per gm. <sup>1</sup>	I. U. per gm.	µg. per gm.
0	..	63	62	3.8	13
4	30	55	58	3.6	12
3	70	33	47	3.8	13
4	98	16	41	3.7	12
6	98	..	33	..	13

<sup>1</sup> Converted from micrograms to International Units by use of the conversion factor 2.

### DISCUSSION

As the time and labor involved in the biological assay method for vitamin A preclude its use in routine analysis, the spectrophotometric method was studied with the intention of correlating the results obtained by the two methods. Preliminary studies of emulsified whole egg and spray-dried egg samples showed that each microgram of vitamin A determined spectrophotometrically was equivalent to two International Units estimated by the biological method. The values in table 1 show reasonably good agreement when the factor 2 is used to convert micrograms to International Units.

The same procedure was followed in estimating the vitamin A content of the stored dried egg, but here the agreement between the two methods was not as good as it was in the case of the recently dried egg samples. The values estimated spectrophotometrically compared less favorably with the biological values as the temperature and time of storage were increased. As high results were obtained with the physical method, presumably the substances responsible for the irrelevant absorption increased in the egg samples during the storage period. Therefore the conversion factor established for the emulsified whole egg and the recently dried egg cannot be applied to stored samples.

As the irrelevant absorption is an additive factor, its subtraction from the total absorption of the vitamin A extract should give the absorption due to the vitamin A. Accordingly an attempt was made to estimate the vitamin A by difference. The results are represented by the absorption curves in figure 1.

Curve 1 is typical of the extract of a dried egg sample. The vitamin A in this extract was destroyed by exposure to sunlight, and the residual absorption measured (curve 2). The difference between these two

curves should be the absorption caused only by the vitamin A. The absorption curve of a vitamin A concentrate was determined (curve 5). The same quantity of the concentrate that was used for this curve was then added to the vitamin free extract; absorption measurements gave curve 3. The vitamin A was destroyed in the extract and also in the solution of vitamin A concentrate. Subsequent absorption measurements resulted in curve 4 for the extract and curve 6 for the concentrate. A comparison of curves 5 and 6 shows that the vitamin was com-

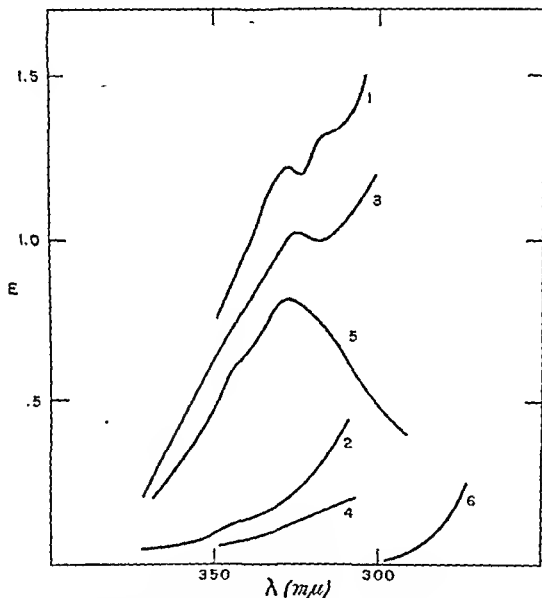


Fig. 1 The effect of irrelevant absorption on the spectrophotometric estimation of vitamin A in stored dried eggs.

- Curve 1 Typical absorption curve of extract of stored dried egg.
- Curve 2 Absorption curve of extract after vitamin A was destroyed by exposure to sunlight.
- Curve 3 Absorption curve of extract after a vitamin A concentrate was added.
- Curve 4 Absorption curve of extract after the added vitamin A was destroyed by exposure to sunlight.
- Curve 5 Absorption curve of a solution of vitamin A concentrate.
- Curve 6 Absorption curve of the solution after the vitamin A was destroyed by exposure to sunlight.

pletely destroyed by exposure to sunlight. As curves 2 and 4 do not show the same intensity of absorption in the region of the vitamin A maximum it is apparent that the irrelevant absorption did not remain constant when the vitamin A was destroyed. Therefore it appears that a conversion factor for each condition of time and temperature of storage would have to be established for the routine analysis of dried egg by the spectrophotometric method.

The retention of vitamin D and riboflavin presents no problem in the keeping of dried egg for the experimental results show that these vitamins are quite stable.

In general the results obtained in this study of the stability of vitamins A, D, and riboflavin in eggs during the spray-drying process and subsequent storage of the dried product are in excellent agreement with the findings of Hauge and Zscheile ('42) and Klose, Jones, and Fevold ('43).

#### SUMMARY

The vitamin A content of emulsified whole eggs and spray-dried eggs was estimated by biological and spectrophotometric methods, the vitamin D content by a biological method and the riboflavin by a microbiological method. The results indicate that no loss of these vitamins occurred during the spray-drying process. Vitamin D and riboflavin are stable in the stored dried products whereas vitamin A is lost fairly rapidly especially at the higher storage temperatures. Estimation of vitamin A in the stored products by the spectrophotometric method presented difficulties. The effect of impurities that absorb in the region of the vitamin A maximum does not remain constant during the storage of the dried product. Attempts to overcome this difficulty are discussed.

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# VITAMIN C CONTENT OF WILD GREENS<sup>1</sup>

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## INTRODUCTION

In a recent publication (Stratton, '43), one of the authors has called attention to a number of native wild plants that are useful additions to war-time diets as greens or salad plants. The present paper reports assays of the vitamin C (ascorbic acid) content of a number of the commonest of these edible wild plants, the tests involving both fresh samples, those which had been placed in frozen storage for several weeks before testing, and those which had been cooked.

The literature records tests of vitamin C content for a few species of wild greens in the fresh state. Burrell and Ebright ('40) report assays for this vitamin in dandelion, shepherd's purse, and pokeweed. Burrell and Miller ('39) have also determined vitamin C content in curly dock, wild lettuce, and chickweed. The values for curly dock and chickweed obtained by these workers were comparable to those obtained in this study, but they reported higher vitamin C values for dandelion, shepherd's purse, and pokeweed than were obtained in our determinations. All of these tests were made by means of the dye titration method.

It is well known that the food value of various crops is markedly affected by the environment and soil in which the crop has grown; for this reason an attempt was made to gather the samples for testing in locations characterized by differences in soil, shading, and other ecological variables.

## MATERIALS AND METHODS

The species of wild plants tested are listed in table 1. An attempt was made to gather and prepare the plant tissues in ways comparable to the recommended practices in gathering and preparing wild greens

<sup>1</sup> The work reported here was carried out under a joint project of the Oklahoma Agricultural Experiment Station, and the School of Arts and Sciences of the Oklahoma Agricultural and Mechanical College.

TABLE 1  
Vitamin C content of fresh, cooked, and frozen wild greens.

SPECIMEN	HABITAT					MG./GM. VITAMIN C, CALCULATED ON:				
Plant	Sample	Shade <sup>1</sup>	Soil <sup>2</sup>	Plowed <sup>3</sup>	Succulence <sup>4</sup>	Wet basis			Dry basis	
						Fresh	Cooked	Frozen	Fresh	Cooked
Curly dock ( <i>Rumex crispus</i> L.)	I	±	L	—	S	1.30	1.30	...	13.83	13.83
	II	±	L	+	MS	0.96	0.90	0.23	8.43	7.90
	III	—	BL	—	MS	1.42	1.43	0.33	9.57	9.67
	IV	—	L	—	S	1.55	1.34	0.49	10.76	9.29
	V	—	SC	—	VS	1.26	1.35	0.53	8.64	9.26
	Mean					1.30	1.26	0.39	10.24	9.99
Prickly lettuce ( <i>Lactuca scariola</i> L.)	I	+	L	—	VS	0.43	0.37	...	6.32	5.42
	II	±	L	—	S	0.44	0.40	...	3.71	3.35
	III	—	L	—	MS	0.60	0.32	0.04	4.58	2.67
	IV	±	L	+	VS	0.31	0.40	0.23	2.64	3.42
	V	—	SC	—	VS	0.31	0.41	0.11	2.83	3.82
	Mean					0.41	0.38	0.12	4.01	3.73
Poverty weed ( <i>Monolepis Nuttalliana</i> (R. & S. Wats.)	I	—	L	+	S	1.18	0.67	...	10.69	6.11
	II	+	CL	—	VS	0.66	0.67	0.13	6.32	6.47
	III	—	L	+	S	0.67	0.67	0.19	4.79	4.79
	IV	—	SC	—	MS	0.81	0.67	0.25	6.11	5.08
	V	—	CL	—	MS	0.67	0.73	0.15	7.50	8.15
	Mean					0.80	0.68	0.18	7.08	6.12
Common chickweed ( <i>Stellaria media</i> (L.) Cyrill.)	I	+	L	—	VS	0.24	0.19	0.20	3.03	2.38
	II	±	CL	—	MS	0.33	0.29	0.02	3.30	2.93
	III	—	SC	—	MS	0.35	0.29	0.05	2.95	2.44
	IV	+	L	—	MS	0.06	0.11	0.15	0.61	1.04
	V	—	SL	+	MS	0.34	0.40	0.17	4.20	3.58
	Mean					0.26	0.26	0.12	2.82	2.47
Common dandelion ( <i>Taraxacum officinale</i> Weber)	I	±	L	—	S	0.19	0.15	...	1.24	1.01
	II	±	SL	—	S	...	0.19	...	...	1.21
	III	—	CL	—	S	0.12	0.32	0.01	0.84	1.10
	IV	+	L	—	VS	...	0.19	0.01	...	1.75
	V	+	CL	—	S	...	0.21	...	...	1.52
	Mean					0.15	0.21	0.01	1.04	1.32
Red-seeded dandelion ( <i>Taraxacum erythrospermum</i> Andr.)	I	±	L	—	S	0.11	0.14	...	0.76	0.99
	II	—	CL	—	S	0.05	0.53	0.07	0.31	0.16
	III	—	CL	—	MS	0.19	0.22	0.23	1.35	1.56
	IV	+	CL	—	S	...	0.19	...	...	1.39
	Mean					0.11	0.27	0.15	0.81	1.02
	Tall dock ( <i>Rumex altissimus</i> Wood)	I	—	BL	—	MS	1.58	1.52	0.17	10.56
II		—	L	—	VS	1.43	1.59	0.72	13.25	14.67
III		—	BL	—	MS	1.36	1.17	0.42	10.59	9.16
IV		—	SL	—	S	1.20	1.31	0.58	9.71	10.57
V		—	SL	—	VS	1.14	1.37	0.30	8.15	9.78
Mean						1.34	1.39	0.44	10.45	10.93
Shepherd's purse ( <i>Capsella Bursa-pastoris</i> (L.) Medic.)	I	—	L	—	MS	0.68	0.64	0.06	6.04	5.00
	II	+	CL	—	MS	0.65	0.93	0.19	4.96	7.04
	III	—	CL	—	MS	0.73	0.91	...	5.05	6.33
	IV	+	CL	—	FS	0.62	0.61	...	4.92	4.69
	Mean					0.67	0.77	0.12	5.24	5.76
	Pokeweed ( <i>Phytolacca americana</i> L.)	I	—	SL	—	S	1.28	1.19	0.28	13.38
II		—	SL	—	S	1.18	1.16	0.85	11.77	11.62
III		±	SL	—	S	1.44	1.75	0.35	13.50	16.55
IV		—	SCL	—	S	1.68	1.70	0.45	15.25	15.40
Mean						1.39	1.45	0.48	13.47	14.00
Lamb's quarter ( <i>Chenopodium album</i> L. I, III, V. <i>Chenopodium leptophyllum</i> Nutt. II, IV.)		I	—	SL	+	VS	0.69	0.99	0.27	5.92
	II	—	SL	—	S	0.51	0.55	...	5.61	6.12
	III	±	SL	—	VS	0.53	0.41	0.16	4.45	3.43
	IV	—	SL	—	S	0.66	0.88	0.34	4.89	6.30
	V	—	SCL	+	S	0.91	0.94	0.41	7.35	7.62
	Mean					0.66	0.75	0.30	5.64	5.64

<sup>1</sup> Shade: +, full shade; ±, partial shade; —, full sun.

<sup>2</sup> Soil: L, loam; B, black; S, sandy; C, clay.

<sup>3</sup> Plowed: +, plowed within past 6 months; —, unplowed during this time.

<sup>4</sup> Succulence: S, succulent; V, very; M, moderately; F, fairly.

for the table. The majority of the samples were collected between 8 and 11 A.M. and prepared for testing immediately thereafter, before the plants had wilted. An aliquot of each sample was blanched for 1 minute in boiling water, quick-frozen without delay, and placed in frozen-locker storage for a period ranging from 36 to 65 days. A second aliquot was macerated in a Waring Blender and tested without further treatment, while a third aliquot of 50 gm. of greens was cooked for 5 minutes in 100 ml. of boiling, distilled water, after which the assay was made on the combined greens and cooking water. The frozen samples were then treated as the fresh, uncooked samples. The Klett-Summers colorimeter was used for the ascorbic acid determinations which were made according to the method of Loeffler and Ponting ('42).

#### DISCUSSION

The vitamin C determinations may be compared with those reported for spinach, in which the vitamin C content is in the approximate range of 0.50 to 0.65 mg. per gm. of greens (Booher, Hartzler, and Hewston, '42). In comparison with this range the wild greens tested fall into three groups: (a) pokeweed, tall dock, and curly dock, with vitamin C contents about double that of spinach; (b) poverty weed, shepherd's purse, and lamb's quarter, which were, roughly, equal to spinach in vitamin C content; and (c) chickweed, common dandelion, red-seeded dandelion, and prickly lettuce with vitamin C values materially lower than spinach.

In general, the changes in vitamin C content following freezing and cooking were comparable to the changes that occur in cultivated greens. With the exception of the red-seeded dandelion the frozen samples showed marked loss in vitamin C content, as is true for spinach. In some cases the vitamin C values obtained for cooked greens were higher than those for comparable samples of uncooked greens. This was particularly true of the two species of dandelion. In some instances no reading was obtained with the raw material, while the cooked material indicated the presence of vitamin C. This may be due to the use of 1% metaphosphoric acid for extraction of the vitamin. Some of these greens may have had higher oxidative activity and Reid ('42) has concluded that higher concentrations of metaphosphoric acid than were used in these tests are necessary for such refractory materials. If this is the case, the vitamin C content of some of our samples may have been materially higher than was brought out by the assays.

The frozen samples varied in their time of frozen storage from 36 to 65 days. Comparison of the loss in vitamin content with length of time

in frozen storage showed no correlation. There was no evidence of a progressive loss in vitamin C content with increasing time of storage within these limits.

The data in table 1 give some indication that plants growing in full sunlight have more vitamin C than those growing in the shade, those in unplowed ground more than those in cultivated land, and those in rich loam or sandy loam soils more than those growing in clay or sandy clay soils. Many factors appear to influence the vitamin C content of wild greens, including soil, moisture, temperature, time of day sample is taken, stage of development of the plant, and others. It was not within the scope of this study to attempt to determine the individual effects of these factors. Such a study would require the growing of plants under controlled conditions.

### SUMMARY

Vitamin C assay of fresh, cooked, and frozen samples of edible wild greens showed that pokeweed, tall dock, and curly dock contained twice as much of this vitamin as spinach; poverty weed, shepherd's purse, and lamb's quarter about as much as spinach; chickweed, dandelions, and prickly lettuce considerably less than spinach. Loss of vitamin C in wild greens due to freezing or cooking was comparable to the loss sustained under similar conditions by cultivated greens. There was some indication that the vitamin C content was greater in plants exposed to full sunlight and in fertile, uncultivated soil.

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# A STUDY OF CANINE HYSTERIA PRODUCED BY FEEDING CERTAIN BAKED DOG FOODS AND WHEAT GLUTEN FLOUR<sup>1</sup>

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## FOUR FIGURES

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The existing knowledge and theories concerning canine hysteria were carefully discussed by Hewetson ('36). He was unable at that time to arrive at any definite conclusions regarding the disease from consideration of the known facts. At best it is an ill-defined malady whose nomenclature, cause, and treatment are in dispute. Numerous other terms have been suggested and used for the syndrome, namely, running fits (Arnold and Elvehjem, '39), fright disease (Grace, '30), epizootic hysteria, enzootic hysteria, infectious hysteria (Campbell, '27), and hyperkinesia (Lintz and Lintz, '29).

The symptoms generally associated with the disease are paroxysmal attacks of hyperexcitability characterized by running, barking or howling, faulty vision, deranged balance, dementia, and often clonic convulsions. The secretion of a heavy mucous saliva is common especially during the convulsions. During the convulsions urination and defecation frequently occur. The affected dogs seem indifferent to injury or to efforts to quiet them. Excitement and physical strain influence the onset and frequency of attacks. Heredity (Berryman and Schlotthauer, '41) is also believed to be a factor affecting the animals' susceptibility. Between attacks the animal may appear normal but is often nervous and apprehensive. Attacks are usually precipitated by stimuli such as a sudden noise or a flash of light.

A number of afflictions have been reported to produce similar symptoms. Melnick and Cowgill ('37) observed hysteria among experimental dogs receiving rations containing gliadin. Arnold and Elvehjem ('39) found that a baked dog food made of wheat flour and meat scrap caused fits in young dogs and thought that a lysine deficiency was involved.

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They also suggested that a toxicity might be a contributing factor. Patton ('39) attributed similar symptoms in dogs fed a baked dog food to a thiamine deficiency. McGhee ('36) thought that the condition was due to a low blood magnesium level. Chambers ('30) reported a case in which the addition of quantities of tallow to a ration of meat and baked bread cured hysteria. Others have believed that distemper (Grace, '30), ear mites (Chambers, '30), intestinal obstruction (Browning, '29), vitamin A deficiency (Walston, '33), or ticks (Macfie, '30) were the cause of the disease.

Many of the workers who believe the disease to be related to the nutrition of the affected animals have commented on its frequent association with the feeding of baked dog foods. For this investigation it was originally intended to select commercial baked dog foods on the basis of their hysteria-producing potency and secure sufficient quantities of the worst offenders for further experimentation. In the midst of this program, Dr. H. B. Parry,<sup>2, 3</sup> who had been conducting feeding experiments with gliadin in this laboratory discovered that by feeding commercially produced wheat gluten to young dogs all of the usual symptoms of canine hysteria could be produced with great rapidity, and intensity. The frequent occurrence of wheat products as an ingredient of baked dog foods, and the similarity of the symptoms suggested that the disease was the same in both cases. The use of wheat gluten offered a more productive approach to the problem than the feeding of commercial dog foods and subsequent efforts were directed to the investigation of this material.

## EXPERIMENTAL

### *Animals*

Mongrel dogs were obtained at weaning and fed a milk diet for several days. During this interval they received as a vermifuge  $\frac{3}{4}$ -grain of santonin and  $\frac{3}{4}$ -grain of phenolphthalein following a 12-hour starvation period, after which they were transferred to a normal, adequate, dry ration for 1 week before being used for experimental purposes. The animals were kept in a heated, lighted room in individual cages of heavy wire mesh, the floors of which were of concrete covered with wood shavings. The proximity of the animals to one another was such as to permit ready transmission of the malady if an infectious agent were responsible.

<sup>2</sup> Unpublished data.

<sup>3</sup> Present address: Imperial Chemical Pharmaceuticals, Entebbe Uganda, British East Africa.

At the end of each experiment animals which had received hysteria-producing rations were transferred to a normal ration for a recovery period of at least 1 week. By this procedure it was possible to use each animal for several experiments. After animals had attained the age of 6 to 8 months their susceptibility was so reduced as to make them unsatisfactory for assay purposes.

*Production of canine hysteria with commercial  
baked dog foods*

The baked dog foods used in this portion of the work were regular commercial products. They were coarsely ground and 97 parts of the ground material mixed with 1 part cod-liver oil, 1 part NaCl, and a part Liver Fraction B<sup>4</sup>. Preliminary experiments had demonstrated that the above supplements did not prevent the occurrence of attacks of canine hysteria. The above rations and water were supplied ad libitum.

TABLE 1  
*Canine hysteria from the feeding of baked dog foods.*

SAMPLE	RESULTS	SAMPLE	RESULTS	SAMPLE	RESULTS
1	fed 18 days; no attacks	6	first attack on 14th day	10	first attack on 24th day
2	fed 34 days; no attacks	8	first attack on 5th day	10	first attack on 11th day
3	fed 41 days; no attacks	7	fed 83 days; no attacks	11	first attack on 32nd day
5	fed 34 days; no attacks	9,	first attack on 5th day	11 plus 5% casein	first attack on 23th day
		9	first attack on 5th day		

Results obtained on nine samples are listed in table 1. Limited quantities of sample 1 prevented a more prolonged assay. Two samples were found to cause attacks in less than a week. Two other samples also caused hysteria but more slowly. Five samples failed to show activity. The affected animals displayed symptoms of nervousness, fright, running, howling, and in some cases convulsions. Secretion of a heavy mucous saliva occurred during attacks. The loss of sight which occurred was not complete nor was it permanent. The animals' pupils were di-

<sup>4</sup> Wilson Laboratories.

lated and the eyes were fixed and staring. No interference with the light reflex was apparent.

The effects of a number of compounds, the lack of which have been associated with seizures of hysteria by other workers, were studied with those samples found to produce attacks. The responses of a test animal receiving sample 6 to supplementation with thiamine hydrochloride, casein, dried canned dog food, and lysine hydrochloride are

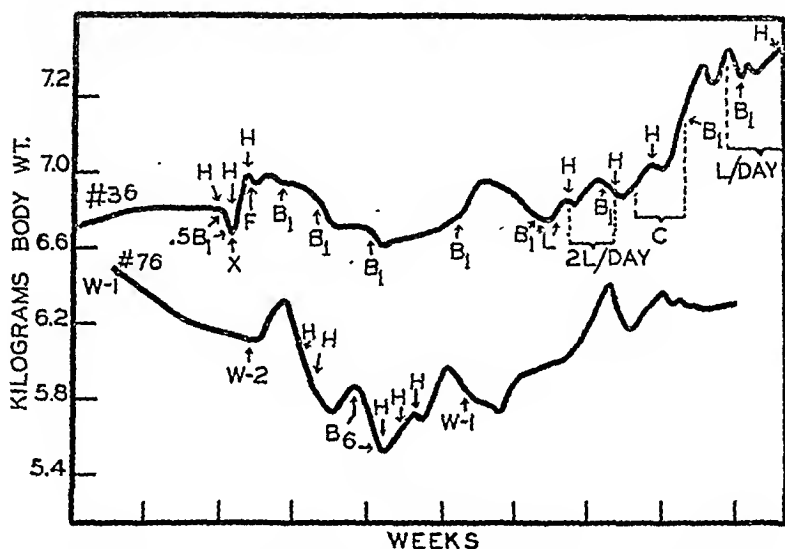


Fig. 1 Growth curves of dogs fed hysteria production rations. H = hysteria attack.

Dog no. 36. Fed dog food sample no. 6 throughout the period shown in the graph. Supplements:  $B_1$  = 4 mg. of thiamine hydrochloride; C = 10 gm. of casein per day; X = 200 gm. of canned dried dog food; F = 135 gm. of canned dried dog food; L = 250 mg. of d-lysine dihydrochloride.

Dog no. 76. Fed ration W-1 (casein ration, see table 2) for 2 weeks before receiving wheat gluten ration W-2 for 3 weeks;  $B_6$  represents oral administration of 2 mg. of pyridoxine hydrochloride.

shown in figure 1 (dog no. 36). None of these supplements either prevented or appreciably delayed the onset of attacks. Sample 11 (table 1) still caused hysteria when supplemented with 5% crude casein. The lysine, canned dried dog food, and casein supplements elicited small growth responses. Similar effects were obtained with other dogs.

### *Canine hysteria from wheat gluten*

Several laboratory rations were devised to study the effects of wheat gluten (table 2). Rations W-1 and W-2 are Dr. Parry's modifications of Melnick and Cowgill's rations ('37). The remainder of the rations

have been modified to correspond more closely to the constituents generally used in this laboratory for experimental animals. To reduce the chances of rancidity not more than a week's supply of each ration was mixed at a time. The dogs were given access to 50 gm. of ration per kilogram of body weight per day, which was approximately the average consumption on rations of this type. This restriction was found necessary to prevent the dogs from over-eating when first fed these high sucrose rations. Such gorging is followed by indigestion and a marked drop in food consumption which may persist for several days. Water was supplied ad libitum. Supplements of proteins and amino acids which amounted to more than 1% of the ration were substituted for equal amounts of sucrose.

TABLE 2  
*Laboratory rations used for wheat gluten studies<sup>1</sup>*

CONSTITUENT	W-1	W-2	W-3	W-4	W-7	W-8	W-9	W-10	W-12
Acid-washed casein . . . . .	4.76	...	.	27.5	20	20	20	20	20
Wheat gluten . . . . .	...	4.37	30	..	10	15	20	25	..
Lard . . . . .	3.77	3.77	21	21	21	21	21	21	21
Sucrose . . . . .	7.50	6.33	40	42.5	40	35	30	25	50
Salts 4 . . . . .	...	..	4	4	4	4	4	4	4
Wesson salts . . . . .	0.30	0.30	..	..	..	..	..	..	..
Bone ash . . . . .	0.40	0.40	..	.	..	..	..	..	..
Liver Fraction B (Wilson) <sup>2</sup> . . . .	0.33	0.33	3	3	3	3	3	3	3
Cod liver oil . . . . .	Note <sup>3</sup>	Note <sup>3</sup>	2	2	2	2	2	2	2
Total . . . . .	17.06	15.90	100	100	100	100	100	100	100

<sup>1</sup> All animals supplemented with 1.5 mg. of thiamine hydrochloride, 2.0 mg. of niacin, 1.0 mg. of pyridoxine hydrochloride, and 10 mg. of alpha-tocopherol per kilogram of body weight per week. Supplements given orally by pipette.

<sup>2</sup> Wilson Laboratories, Chicago, Ill.

<sup>3</sup> Two ml. cod liver oil orally per day by pipette.

*Experiment I.* Two 6-month-old fox terriers weighing 5.7 kg. and 6.5 kg. were fed ration W-1 (casein) for 13 days. The ration was changed to W-2 (wheat gluten) and the incidence of hysteria attacks observed. The responses of animal no. 76 are shown in figure 1. This dog had an attack after 5 days on the wheat gluten ration. The other suffered from deranged balance by the eighth day and had attacks of hysteria on the tenth day, dying after 22 days on the ration. Supplements of additional crystalline pyridoxine were given orally to both animals without effect.

*Experiments II, III, IV. Effects of lysine and protein supplements. II.* (fig. 2). Six pups 7 weeks of age were fed ration W-1 (casein) for 9

days. Dogs nos. 78, 80, 81 and 82 were then changed to ration W-2 (wheat gluten). In addition, dogs nos. 81 and 82 received d-lysine dihydrochloride<sup>5</sup> equivalent to 0.6% of the ration. Dogs nos. 79 and 83 were maintained on the casein ration as controls. Control dogs nos. 79 and 83 continued to grow at a good rate and were normal in behavior. The four littermates fed rations containing wheat gluten all had attacks of hysteria 3 days after the change in rations. Six-tenth per cent d-lysine dihydrochloride in the diet did not retard the onset of attacks.

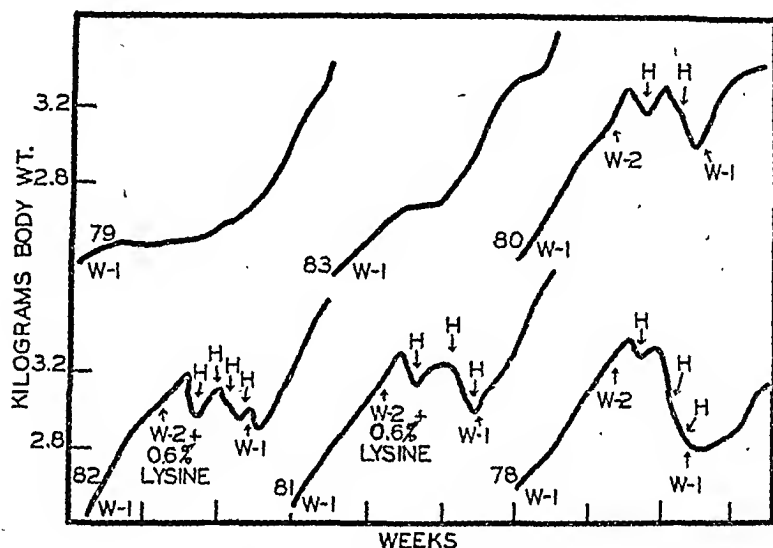


Fig. 2 Supplementation of wheat gluten ration with lysine. Control dogs, no. 79 and no. 83, fed casein ration W-1 throughout experiment. H = hysteria attack.

III. (figs. 3a, 3b). After a 12-day recovery period on ration W-1 dogs nos. 78, 79, 80, 81, 83 and 85 were placed on the following experiment. No. 78 was maintained on the casein ration as a control. No. 81 was fed ration W-2; nos. 79, 80, 81 and 85 received W-2 plus the supplements designated on the graphs. Neither 1.2% d-lysine dihydrochloride, 18% casein, 18% casein hydrolysate, nor 18% gelatin prevented hysteria. The onset of the disease seemed to be slightly delayed in the dogs receiving the protein supplements and the growth rate was increased by the addition of casein or casein hydrolysate. Calcium pantothenate (fig. 3b) did not alleviate the symptoms.

IV. After a 9-day preliminary feeding period on ration W-4 (casein), two black Irish-Setter-German Shepherd Cross littermates, nos. 90 and 91, were fed ration W-3 (wheat gluten). The ration of no. 91 was

<sup>5</sup> Merck.

supplemented with 4.97% dl-lysine monohydrochloride. Dog no. 90 developed hysteria in 2 days; dog no. 91 had its first attack in 3 days. The growth of the animal fed lysine was slightly better.

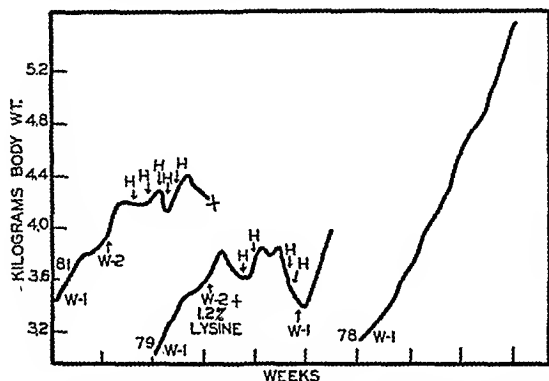


Fig. 3a Effect of supplementing wheat gluten rations. Supplements of 2.5, 2.0 and 10.0 mg. of calcium pantothenate were administered orally to dog no. 79 during the seventh, eighth, and tenth days on ration W-2 plus 1.2% d-lysine dihydrochloride. Dog no. 81 in a similar manner was fed 2.5, 5.0, and 10.0 mg. of calcium pantothenate during the ninth, eleventh, and twelfth days on ration W-2. H = hysteria attack.

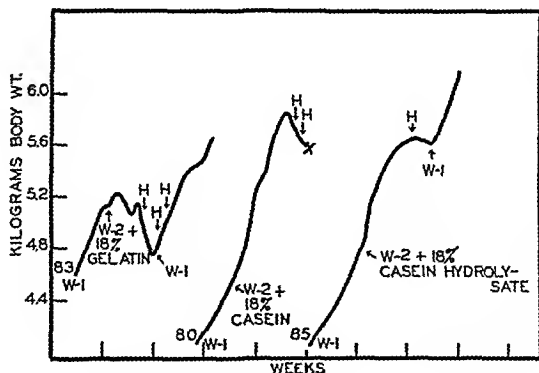


Fig. 3b Effect of supplementing wheat gluten rations. H = hysteria attack.

The disease does not seem related to pyridoxine-deficiency fits of rats and pigs reported by Chick ('40), nor the valine-deficiency staggers of rats observed by Rose ('39).

The nature of the toxic factor is still in question. It appears to be distinct from selenium which has been found to be the toxic substance in certain grains (Moxon, '37). Recent analyses made by Dr. Moxon of the South Dakota Experiment Station show the selenium contents of two samples of our wheat gluten flour to be 1.2 and 2.4 parts per million. Even when these materials were fed as 30% of the rations the intake of selenium was well below the minimum levels reported to be toxic. Balls and Hale ('40) have reported a material extracted from wheat flour by petroleum ether and found to be toxic (Coulson et al., '42) for mice and certain microorganisms. We have not found wheat gluten flour toxic for mice when fed as 30% of the ration.<sup>7</sup>

As a further point of interest it should be mentioned that in preliminary studies we have been unable to detect any of the usual symptoms of canine hysteria in the chick, rat, guinea pig and monkey when fed rations containing large amounts of wheat gluten, for periods of 3 weeks or more.<sup>7</sup> Melnick and Cowgill ('37) have also reported the rat to be immune to the toxic factor in gliadin.

The chemical properties of the toxic material in wheat gluten are now under investigation. Although details of the work cannot be presented here, it is significant that the wheat gluten can be rendered water-soluble by digestion with pancreatin.<sup>7</sup> It does not seem probable that it can be causing any physical interference with gastro-intestinal function in spite of its extremely gummy character previous to digestion.

#### SUMMARY

Canine hysteria of the nutritional type has been produced in dogs by feeding baked dog foods containing wheat gluten flour and experimental rations containing wheat gluten flour. The disease appears to be caused by some toxic factor present in the wheat products. Although the proteins of these products are deficient in lysine, protein and lysine supplements do not prevent the disease. Hysteria can be caused in dogs receiving nutritionally adequate rations by the addition of wheat gluten flour to the rations.

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## EDITORIAL REVIEW

### THE ATWATER SYSTEM OF CALCULATING THE CALORIC VALUE OF DIETS

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Some years ago Morey ('36) made a very comprehensive analysis and comparison of methods of calculating the energy value of diets, from which she concluded: "The multiplicity of methods used in calculating the amount of energy actually derived by the body from a given amount of food eaten or purchased makes it extremely difficult to compare accurately different dietary studies and standards." The problems here involved become of special importance at the present time because of the need for a common basis for international comparisons of food supplies and for their evaluation in relation to dietary needs. This review deals with these problems, with particular reference to the applicability of the Atwater system.

In this system, which has long been generally employed in the United States, calories are obtained by multiplying grams of protein, fats and carbohydrates by the factors, 4-9-4, respectively. On the other hand the Rubner factors, 4.1-9.3-4.1, have generally been used in Europe and elsewhere. The principal difference between the two sets of factors is that the Atwater values take account of digestion losses which those of Rubner do not. Atwater has pointed out that Rubner's values for fat and carbohydrate are heats-of-combustion values and that when they are multiplied by the appropriate coefficients of digestibility they become approximately the same as his own (Atwater's). Applied to mixed diets Rubner's factors give a caloric value which is about 3% higher than obtained by Atwater's factors, according to Morey ('36) who has reviewed the bases of the two sets of values.

There is a further difference in the practice in the U.S.A. and in the U.K. (United Kingdom). According to the Atwater system the carbohydrate factor 4 is applied to carbohydrates as determined "by difference", whereas the British workers, McCance and Widdowson ('42)

make the calculation on the basis of what they designate as the "available carbohydrate", namely, the directly determined starch, sugars and dextrins. Further they express all of these carbohydrates as glucose and multiply the total by 3.75, the heat-of-combustion value of this sugar, to obtain calories.

More recently the Accessory Food Factors Committee of the Medical Research Council of the U.K. have used the Atwater factors in computing the composition of war-time foods but they have expressed the "available carbohydrates" in terms of their starch equivalents (disaccharides  $\div$  1.055, hexoses  $\div$  1.11) before applying the factor 4, whereas by the Atwater system the total carbohydrates are multiplied by the factor directly. Thus the M.R.C. (Medical Research Council) procedure results in lower values for all foods containing sugars. Cane sugar, for example, has approximately 5% fewer calories by the M.R.C. calculation than by the Atwater method.

The problems arising from the discrepancies between the U.K. procedure and the Atwater system as used in the U.S.A. and Canada became apparent in a joint study made by experts of the three countries, involving a comparison of food consumption levels in relation to nutritional needs. The difficulties encountered are discussed in Appendix D of the Report of the Special Joint Committee ('44). The problem has arisen again in studies in which U.K. and U.S.A. scientists are cooperating in evaluating the food supplies of various countries of continental Europe, as well as in connection with the activities of the United Nations Relief and Rehabilitation Administration (U.N.R.R.A.). It will continue to be a problem, post-war, for international programs in the field of food and nutrition unless a common basis for food evaluation is agreed upon. The present article is presented as a contribution to this objective. The experimental basis of the Atwater system is reviewed, with particular reference to the points of difference with the present U.K. procedure. Its applicability to diets differing widely in make-up from the U.S. diet is critically discussed.

Atwater ('00) has summarized the experimental basis of his procedure in the 12th Annual Report of the Storrs Agricultural Experiment Station. The detailed supporting data are published in other reports from this station, in bulletins of the Office of Experiment Stations and elsewhere. For purposes of the present study and because the publication in question is doubtless unavailable in many libraries, table 10 from the Storrs Report is here reproduced without change (table 1).

Atwater first summarized 185 dietary studies made on different groups of people in different areas of the U.S.A. He classified the foods

TABLE 1

*Factors for heats of combustion and fuel values of nutrients in different groups of food materials and in mixed diet.*

(Table 10 from Atwater, Report of Storrs Agricultural Experiment Station for 1899.)

KIND OF FOOD MATERIAL	NUTRIENTS FURNISHED BY EACH GROUP FOR 100 GRAMS TOTAL	HEAT OF COM- BUSTION PER GRAM	PRO- PORTION OF TOTAL NUTRIENT ACTUALLY AVAILABLE	TOTAL ENERGY PER GRAM IN AVAILABLE NUTRIENTS	FUEL VALUE	
					Per gram available nutrients	Per gram total nutrients
	A	B	C	D =(B × C)	E <sup>1</sup>	F <sup>2</sup>
<i>Protein</i>	<i>Gm.</i>	<i>Cal.</i>		<i>Cal.</i>	<i>Cal.</i>	<i>Cal.</i>
Meats, fish, etc.	43.0	5.65	.97	5.50	4.40	4.25
Eggs	6.0	5.75	.97	5.60	4.50	4.35
Dairy products	12.0	5.65	.97	5.50	4.40	4.25
Animal food	61.0	5.65	.97	5.50	4.40	4.25
Cereals	31.0	5.80	.85	4.95	4.55	3.70
Legumes	2.0	5.70	.78	4.45	4.45	3.20
Vegetables	5.5	5.00	.83	4.15	3.75	2.90
Fruits	0.5	5.20	.85	4.40	3.95	3.15
Vegetable food	39.0	5.65	.85	4.80	4.40	3.55
Total food	100.0	5.65	.92	5.20	4.40	4.00
<i>Fat</i>						
Meat and eggs	60.0	9.50	.95	9.00	9.50	9.00
Dairy products	32.0	9.25	.95	8.80	9.25	8.80
Animal food	92.0	9.40	.95	8.95	9.40	8.95
Vegetable food	8.0	9.30	.90	8.35	9.30	8.35
Total food	100.0	9.40	.95	8.90	9.40	8.90
<i>Carbohydrates</i>						
Animal food	5.0	3.90	.98	3.80	3.90	3.80
Cereals	55.0	4.20	.98	4.10	4.20	4.10
Legumes	1.0	4.20	.97	4.05	4.20	4.05
Vegetables	13.0	4.20	.95	4.00	4.20	4.00
Fruits	5.0	4.00	.90	3.60	4.00	3.60
Sugars	21.0	3.95	.98	3.85	3.95	3.85
Vegetable food	95.0	4.15	.97	4.00	4.15	4.00
Total food	100.0	4.15	.97	4.00	4.15	4.00

<sup>1</sup> Values for fats and carbohydrates, same as corresponding values in column B. Values for protein, same as corresponding values in column B minus 1.25.

<sup>2</sup> Values for fats and carbohydrates, same as corresponding values in column D. Values for protein, same as corresponding values in column D minus 1.25.

make the calculation on the basis of what they designate as the "available carbohydrate", namely, the directly determined starch, sugars and dextrins. Further they express all of these carbohydrates as glucose and multiply the total by 3.75, the heat-of-combustion value of this sugar, to obtain calories.

More recently the Accessory Food Factors Committee of the Medical Research Council of the U.K. have used the Atwater factors in computing the composition of war-time foods but they have expressed the "available carbohydrates" in terms of their starch equivalents (disaccharides  $\div$  1.055, hexoses  $\div$  1.11) before applying the factor 4, whereas by the Atwater system the total carbohydrates are multiplied by the factor directly. Thus the M.R.C. (Medical Research Council) procedure results in lower values for all foods containing sugars. Cane sugar, for example, has approximately 5% fewer calories by the M.R.C. calculation than by the Atwater method.

The problems arising from the discrepancies between the U.K. procedure and the Atwater system as used in the U.S.A. and Canada became apparent in a joint study made by experts of the three countries, involving a comparison of food consumption levels in relation to nutritional needs. The difficulties encountered are discussed in Appendix D of the Report of the Special Joint Committee ('44). The problem has arisen again in studies in which U.K. and U.S.A. scientists are cooperating in evaluating the food supplies of various countries of continental Europe, as well as in connection with the activities of the United Nations Relief and Rehabilitation Administration (U.N.R.R.A.). It will continue to be a problem, post-war, for international programs in the field of food and nutrition unless a common basis for food evaluation is agreed upon. The present article is presented as a contribution to this objective. The experimental basis of the Atwater system is reviewed, with particular reference to the points of difference with the present U.K. procedure. Its applicability to diets differing widely in make-up from the U.S. diet is critically discussed.

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Atwater first summarized 185 dietary studies made on different groups of people in different areas of the U.S.A. He classified the foods

mon vegetables. That the carbohydrate represented by these differences is entirely unavailable has not been proved.

It has been mentioned that the Atwater factors for a mixed diet do not apply accurately to many individual foods. While the factor 4 applies to the mixture of proteins in the U.S. diet, the value for eggs is 4.35, meat 4.25, cereals 3.7, legumes 3.2, etc. Similarly the carbohydrate factors for individual foods range from 4.1 for cereals to 3.6 for fruits. Thus, the fuel values for certain individual foods as they appear in tables of food composition, calculated by the use of the average factors, 4-9-4, can be considered only approximations. The errors here tend to balance out in calculating the fuel values of mixed diets similar to the average diet upon which the factors are based and thus are of no great concern. But this is not true for certain food combinations and the errors involved in the individual values should be recognized in connection with controlled experiments with diets which vary markedly in make-up from the pattern on which the overall values, 4-9-4, are based. For example, if one desired to compare a diet of animal products with a diet of cereals, legumes, and vegetables, at the same calorie level, and used the factors, 4-9-4, for both combinations, the desired calorie equality would not be achieved.

The real problem in the use of the Atwater factors arises in connection with diets which are markedly different in food make-up from the average mixed diet upon which they are based. Compared with the latter, the U.K. wartime diet is lower in animal products and higher in potatoes and cereals. Thus the Atwater factors would tend to overvalue it because animal protein has a higher fuel value than vegetable protein. The cereal carbohydrate of the Atwater diet came very largely from highly milled flour, as is the case for the present U.S.A. diet. The flour of the U.K. wartime diet, however, is an 85% extraction, and its energy is somewhat less digestible than the highly milled products.

Thus, on this basis, the Atwater factors tend to overvalue the calories of a diet such as the present U.K. combination which contains over one-third of its calories from this somewhat less digestible source. These considerations undoubtedly influenced U.K. authorities in adopting their present procedure. The overvaluation which might result from the use of the factors, 4-9-4, becomes of greater concern in connection with the wartime diets of occupied countries which consist much more largely of foods of plant origin, and particularly of less refined foods higher in fiber and thus less digestible. For the same reasons their application to the usual diets of Eastern peoples is subject to error.

Atwater never intended that the factors, 4-9-4, should be applied without modification to all diets. His data reproduced in the table provide the basis for deriving other sets of overall factors for any combination of the food groups and thus for any diet made up largely of the groups listed. As an alternative, the individual fuel value for each nutrient of each food or food group, as listed in column F, could be applied to the corresponding nutrient supply data to arrive at caloric values by foods or food groups, and their values could then be totalled to give the calories for the diet as a whole. In this way the Atwater system could be applied to any diet, without introducing the errors inherent in the average factors for diets differing widely from the original average mixed diet. For either of these procedures a figure for vegetable fats as such, not provided by the Atwater table, is required. It would seem that they should be assigned the fuel value of 9, the same as animal fats, rather than the figure of 8.35 which Atwater lists for fats in "vegetable food". Further, factors would need to be provided for cereals of low extraction. There are digestion data in the literature which could furnish the basis for such factors.

The above procedures would of course greatly increase the calculations required compared with the present use of a single set of factors for the diet as a whole. A short-cut procedure which would correct for significant deviations in the proportions of foods of plant and animal origin from the pattern of the average mixed diet would involve a re-weighting of the average values accordingly. Atwater's overall fuel value of 4 for protein is based upon an approximately 60:40 distribution of animal protein averaging 4.25 cal. per gram and of vegetable protein averaging 3.55 cal. A 30:70 distribution would give an overall value of 3.75. Thus, for any diet an appropriate overall value for protein could be calculated by weighting Atwater's values for the two classes of protein in accordance with the proportions of each present. Similarly, appropriate values could be computed for fat and carbohydrates. This procedure would not take account of variations caused by large and significant differences in food distribution within the two groups.

Thus, another alternative might involve a condensation of the data of the groups of the original Atwater table to provide values such as those listed in table 2. Here values for cereals of 85% extraction have been included, based upon digestion studies of the U.K. wartime flour. In applying the data in this table to the food supply for a given country, the overall value for calculating its protein calories could be easily derived as an average of the individual values, weighted in accordance

with the proportions of the different classes of foods present. Similarly, an appropriate value for fat could be obtained. It would be little different from the present Atwater value because such a small portion of fat is supplied from foods other than animal products and animal and vegetable fats as such. Similarly, an appropriate value to be applied to "total" carbohydrates would not be significantly lower than 4, except for diets, for which a substantial proportion of the carbohydrate calories is furnished by cereals of low extraction. The table could be condensed, or made more detailed. A study of Atwater's basic data might modify some of the values. They serve here merely to illustrate one possible solution of the problem.

TABLE 2

*Condensation of original Atwater table of fuel values of nutrients in different groups of food materials.*

	PROTEIN	FAT	TOTAL CARBOHYDRATES
Animal products	4.25	9.00	3.8
Animal and vegetable fats		9.00	
Cereals (70% extr.)	3.70 —	8.35	4.1
(85% extr.)	3.60		3.8
Pulses and nuts	3.20 —		4.0
Vegetables (including potatoes)	2.90		4.0
Fruits	3.15 —		3.6
Sugar (sucrose)			3.85

Any of these procedures could be modified to utilize the "available carbohydrate" basis, and the gross caloric values in column E of Atwater's table, if that were deemed desirable. For reasons discussed in this review the writer does not favor the adoption of this basis at the present. Particularly, certain procedures employed in its use by the M.R.C. seem questionable if the experimental basis of the Atwater system is accepted.

The possible procedures here outlined for adapting the Atwater system to diets differing widely from those upon which the factors, 4-9-4, were derived increase the computations which must be made. How far it is worthwhile to attempt to refine the original procedure, in view of errors inherent in estimating food supplies and of the variability represented in data as to "average composition", may be questioned. But for international comparisons, in which there are wide differences in the character of the food supplies, some modifications from the use of a single set of overall factors, but still having a common basis, seem essential. Unless there is a comparable basis for computing caloric



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## MEAD JOHNSON AND COMPANY 'B-COMPLEX' AWARD

Nominations are solicited for the 1945 Award of \$1,000 established by Mead Johnson and Company to promote researches dealing with the B complex vitamins. The recipient of this Award will be chosen by a Committee of Judges of the American Institute of Nutrition and the formal presentation will be made at the annual meeting of the Institute at Cleveland on May 8, 1945.

The Award will be given to the laboratory (non-clinical) or clinical research worker in the United States or Canada who, in the opinion of the judges, has published during the previous calendar year January 1st to December 31st the most meritorious scientific report dealing with the field of the 'B-complex' vitamins. While the award will be given primarily for publication of specific papers, the judges are given considerable latitude in the exercise of their function. If in their judgment circumstances and justice so dictate, it may be recommended that the prize be divided between two or more persons. It may also be recommended that the award be made to a worker for valuable contributions over an extended period but not necessarily representative of a given year. Membership in the American Institute of Nutrition is not a requisite of eligibility for the award.

To be considered by the Committee of Judges, nominations for this award for work published in 1944 must be in the hands of the Secretary by January 15th, 1945. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate the task of the Committee of Judges in its consideration of the nomination.

ARTHUR H. SMITH  
*Wayne University College of Medicine*  
*Detroit, Michigan*

SECRETARY, AMERICAN INSTITUTE OF NUTRITION

## BORDEN AWARD IN NUTRITION

The American Institute of Nutrition will make this award in recognition of distinctive research by investigators in the United States and Canada which has emphasized the nutritive significance of the components of milk or of dairy products. The award will be made primarily for the publication of specific papers, but the judges may recommend that it be given for important contributions over an extended period of time. The award may be divided between two or more investigators. Employees of the Borden Company are not eligible for this honor.

The formal presentation will be made at the annual meeting of the Institute at Cleveland, May 8, 1945. To be considered for the award, nominations must be in the hands of the Chairman of the Nominating Committee by January 15, 1945. The nominations should be accompanied by such data relative to the nominee and his research as will facilitate consideration for the award.

FREDERICK J. STARE  
*Harvard Medical School*  
*Boston, Massachusetts*

CHAIRMAN, NOMINATING COMMITTEE

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## ERRATUM

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Karel, Leonard and C. W. Chapman

The vitamin C level of the blood plasma in guinea pigs.

Footnote 1; page 92, in the legend below table 2 the word "not" which follows "a probability of" should be eliminated.

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